ISOLATION AND CHARACTERIZATION OF SERUM CHYMOTRYPSIN INHIBITOR

ABSTRACT

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A B S T R A C T

Human blood contains several inhibitors of proteolytic enzymes which are implicated in the regulation of physiological processes such as blood coagulation, complement fixation and response to inflammation. These inhibitors exert their regulatory functions by maintaining a proper proteinase-proteinase inhibitor equilibrium in the tissue. Alpha$_1$-antichymotrypsin is one of the important human plasma/ serum inhibitor which inhibits serine proteinases. In view of the desirability of getting relevant informations on alpha$_1$-antichymotrysin from mammals other than human and the fact that inhibitor was found to be absent in certain mammalian serum e.g. porcine, it was thought worthwhile to isolate and characterize the inhibitor from hitherto uninvestigated source i.e. goat.

Goat chymotrypsin inhibitor was isolated both from serum and plasma and their properties were found to be indistinguishable. Goat plasma showing 5% inhibitory activity per milligram of protein was salt fractionated and further purified by ion exchange chromatography on DEAE-cellulose column (2.4 x 6.0 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM NaCl and 0.02% sodium azide. The protein was eluted

batchwise with the buffer containing varying concentrations of sodium chloride (0.1 M - 0.2M NaCl). The protein fractions eluted with the buffer containing 150 mM sodium chloride, showing optimal antichymotryptic activity, were pooled and rechromatographed on the same ion exchange column. The final protein yield of the inhibitor was 0.15% (W/W). Thus about 80 mg of the pure inhibitor was isolated from the goat plasma containing 53 g of protein by the procedure used in this study.

The goat inhibitor preparation moved essentially as a single protein band in 12% polyacrylamide gel during electrophoresis performed in Tris glycine buffer (25 mM Tris and 194 mM glycine), pH 8.3, containing 0.1% SDS with a relative mobility of 0.26. This showed that the preparation of goat chymotrypsin inhibitor obtained in this study was homogeneous with respect to size. The size homogeneity was also indicated by gel filtration on Sephadex G-200 column to be described below.

When the measured relative mobility (0.26) of the chymotrypsin inhibitor was compared with the relative mobilities of marker proteins viz, transferrin, bovine

serum albumin, Ig G, ovalbumin, chymotrypsinogen A and cytochrome c which were electrophoresed under conditions identical to that used for the electrophoresis of the inhibitor, the molecular weight of the inhibitor was found to be 68 kDa. The observation that relative mobiliity of the chymotrypsin inhibitor remained unaltered under reducing (0.02 M 2-mercaptoethanol) and nonreducing conditions taken together with the conclusion that the native molecular weight (76 kDa) as determined by gel filtration suggested that the inhibitor consisted of a single polypeptide chain. The native molecular weight (76 kDa) as determined by gel filtration under native condition is about 12% higher than that (68 kDa) determined by polyacrylamide gel electrophoresis under denaturing conditions. The discrepancy has been ascribed to the glycoprotein nature of the inhibitor.

Goat chymotrypsin inhibitor was found to be a sialoglycoprotein containing 4.5% sialic acid and 12% neutral hexose; these results would mean that the inhibitor molecule contains 10 moles of sialic acid and 47 moles of neutral hexose.

The number of free sulfhydryl group in the goat chymotrypsin inhibitor was determined by titration with p-hydroxymercuribenzoate in 10 mM sodium phosphate buffer, pH 7.0. From the curve between increase in absorbance at 250 nm and molar ratio of p-hydroxymercuribenzoate to inhibitor, the number of free sulfhydryl group per mole of inhibitor was calculated as one. Interestingly, the goat chymotrypsin inhibitor with sulfhydryl group blocked with p-hydroxymercuribenzoate retained its full antichymotryptic activity suggesting that the lone titrable sulfhydryl group in the inhibitor had no functional role.

The isoionic preparation of goat chymotrypsin inhibitor was prepared by passing extensively dialyzed inhibitor solution through Dintzis column. The pH of the inhibitor solution containing 1.6 mg/ml protein was determined to be 5.5. The concentration of isoionic inhibitor solution was measured by dry weight method. The goat inhibitor solution (0.11 mg/ml) absorbed maximally at 278 nm in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. Fluorescence excitation and emission spectra of the goat inhibitor were obtained in the same buffer but at relatively lower protein concentration (0.02 mg/ml). The emission and

and excitation maxima were found to occur near 338 and 278 nm, respectively. These spectral features suggest that inhibitor is a tryptophan containing protein.

The specific extinction coefficient of goat chymotrypsin inhibitor was computed from the measured optical densities of protein solutions of known concentrations. The values of specific extinction coefficient were found to be 6.23 and 5.92 cm^2g^{-1} respectively at 278 and 280 nm.

The hydrodynamic properties of chymotrypsin inhibitor were studied by analytical gel chromatography on a Sephadex G-200 column (2.4 x 78 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02% sodium azide. The column was calibrated with marker proteins of known molecular weights and Stokes radii. These were bovine serum albunim, ovalbumin, pepsin, chymotrypsinogen A and cytochrome c. The elution volume of the goat chymotrypsin inhibitor from the column was measured to be 202 ml which would correspond to a molecular weight of 76 kDa and Stokes radius of 3.53 nm. The Stokes radius of the inhibitor was used in the calculation of its diffusion coefficient and frictional ratio which were found to be 6.29 x 10^{-7}

cm^2 / sec and 1.32, respectively. Thus the measured frictional ratio (1.32) is significantly different than that expected for a globular protein. Alternatively this may be due to unusually high degree of hydration of the goat sialoglycoprotein chymotrypsin inhibitor containing as high as 17% carbohydrate.

The thermal stability of the goat chymotrypsin inhibitor was studied in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride in the temperature range, 37°-60°C. The inhibitor was kept at a given temperature for 15 minutes and its inhibitory activity was measured against bovine chymotrypsin at 37°C. The inhibitor incubated at 37°C and 45°C showed the same inhibitory activity. However, by treatment of the inhibitor at 50°C and 55°C, the losses in inhibitory activity were 35% and 65% respectively. When the inhibitor was incubated at 60°C for 15 minutes and its inhibitory activity was measured, no activity was detected suggesting the inactivation of the inhibitor at 60°C.

Unlike human $alpha_1$-antichymotrypsin the goat chymotrypsin inhibitor was able to inhibit even trypsin

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Medicine of the Aligarh Muslim University

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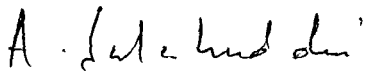
CERTIFICATE

I certify that the work presented in the following pages has been carried out by Mrs. **Renu Tyagi** and that it is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.



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Renu Tyagi.

ALIGARH **[RENU TYAGI]**

Dated: 12/6/89

D E D I C A T E D

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LIST OF ABBREVIATIONS

α-CPI	Alpha-cysteine proteinase inhibitor
α_1-Achy	Alpha$_1$-antichymotrypsin
α_1-AT	Alpha$_1$-antitrypsin
α_1-PI	Alpha$_1$-proteinase inhibitor
α_2-AP	Alpha$_2$-antiplasmin
α_2-M	Alpha$_2$-macroglobulin
AT-III	Antithrombin III
β_1-AC	Beta$_1$-anticollagenase
CI-Inh	CI-Inhibitor
DEAE - Cellulose	Diethylaminoethyl-cellulose
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
IαI	Inter alpha$_1$-trypsin inhibitor
k_{cat}	Catalytic rate constant
kDa	Kilodalton
K_m	Michaelis constant
NaOH	Sodium hydroxide
PAGE	Polyacrylamide gel electrophoresis
pCMB	p - Chloromercuribenzoate
pHMB	p - Hydroxymercuribenzoate

R_m	Relative mobility
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N' - tetramethylethylene diamine
TLCK	N -α- p - tosyl - L - Lysyl - chloromethyl ketone
Tris	Tris (hydroxymethyl) amino methane

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INTRODUCTION

Proteinase inhibitors exist in multiple forms in numerous tissues of animals (Laskowaski and Kato,1980). Their main function appears to be prevention of unwanted proteolysis, however, the exact function of a particular inhibitor is not always known with precision. Many proteolytic processes such as blood coagulation, blood clot dissolution, hormone production or elimination and complement fixation occur in blood and require exquisite control. In vitro proteolytic activities can be regulated by changing pH, temperature, ionic strength and enzyme and substrate concentrations, however, such variables can not operate to control the kinetic equilibria in vivo. Therefore, to control proteolytic activities in vivo a large number of proteinase inhibitors are present in plasma. Proteinase inhibitors were detected in human plasma as far as back in 1894 by Fermi and Pernossi. These inhibitors constitute about 10% of the total plasma proteins (Bodmer and Schnebli, 1984) and form, by weight, the third major component of the functional plasma proteins; the other two being albumin and immunoglobulins (Travis and Salvesen, 1983).

The proteinase inhibitors isolated and characterized from human plasma are listed in Table I. All these inhibitors are glycopeptide in nature with their molecular weights ranging from 54,000-725,000. The inhibitor preparations from plasma and serum are indistinguishable in their molecular and physical properties. These inhibitors differ mainly in their target specificity. Among these, α_2 M inhibits the majority of plasma proteinases from all four catalytic classes of enzymes - serine, cysteine, aspartic and metallo proteinases,while other inhibitors are class specific. Alpha$_1$-antichymotrypsin*, α_1 - PI, Cl-Inh, AT III and IαI inhibit only serine proteinases, α-CPI inhibits only cysteine proteinases and β_1-AC inhibits only collagenolytic enzymes of metalloenzyme class (Travis and Salvesen, 1983).

The same enzyme may be inhibited by more than one inhibitor and each inhibitor can inhibit the activity of more than one enzyme although with varying specificity and avidity. This extraordinary cross coverage serve as

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* In case of goat the proteinase inhibitor acting on bovine chymotrypsin has been referred to as chymotrypsin inhibitor.

TABLE I

PROTEINASE INHIBITORS PRESENT IN HUMAN PLASMA

Name	Molecular weight	Concentration	
		g/litre	M
$Alpha_1$-antichymotrypsin[a]	68,000	0.5	7.7
$Alpha_1$-proteinase inhibitor[a]	55,000	1.3	23.6
Antithrombin - III[a]	62,000	0.29	4.7
$Alpha_2$-macroglobulin[a]	725,000	2.5	3.5
Inter - $alpha_1$- trypsin inhibitor [a]	160,000	0.5	3.1
CI - inhibitor[a]	105,000	0.18	1.7
$Alpha_2$-antiplasmin[a]	67,000	0 07	1.1
$Beta_1$-anticollagenase[b]	30,000-33,000	-	-
Alpha-cysteine proteinase inhibitor[b]	57,000-175,000	-	-

References:- [a] Harpel and Brower (1983)
[b] Travis and Salvesen (1983)

a backup safety system especially in those cases where principal inhibitor is depleted or absent either congenitally or in an acquired way (Murano, 1985). The spectrum of inhibition of the various inhibitors is summarized in Table II. Accordingly it is difficult to identify the target enzyme for a particular inhibitor, however, the usual criteria to identify the target enzyme is to study the rate of complex formation of the inhibitor with suspected enzymes. The enzyme which reacts faster with inhibitor will probably be the target enzyme. This, together with the knowledge of the distribution of enzyme among all the inhibitors in plasma, would substantially reduce the false interpretations with regard to the specific role of a particular inhibitor. However, by using the formula given by Beith (1980), it is possible to measure in vivo half time of inhibition as follows--

$$t_{\frac{1}{2},ass} \simeq 1/k_{ass}[I] \qquad (1)$$

where k_{ass} is the second order rate constant for association of inhibitor and enzyme and [I] is the normal concentration of inhibitor in plasma.

TABLE II *

SPECTRUM OF INHIBITION OF PROTEINASE INHIBITORS

Enzyme		Inhibitor					
		α_1-Achy	α_1-PI	AT III	α_2-M	α_2-AP	CI-Inh
A)	Coagulation --						
i)	Factor XII a	-	-	±	-	±	+
ii)	Factor XI a	-	+	±	-	±	+
iii)	Factor X a	-	+	+	-	±	-
iv)	Factor IX a	0	-	±	0	-	0
v)	Factor VIII	0	0	0	0	0	0
vi)	Factor VII a	0	0	0	0	0	0
vii)	Factor V	0	0	0	0	0	0
viii)	Thrombin	-	+	+	+	-	-
B)	Fibrinolysis --						
i)	Plasmin	-	+	±	+	+	+
ii)	Urokinase	0	±	±	+	±	0
C)	Complement --						
i)	Clr	-	-	-	-	0	+
ii)	Cls	-	-	-	-	0	+
D)	Fertilization --						
i)	Acrosin	-	+	+	+	0	-

Enzyme		Inhibitor					
		α_1-Achy	α_1-PI	AT III	α_2-M	α_2-AP	CI-Inh
E)	Pancreatic hydrolysis --						
i)	Trypsin	-	+	+	+	0	±
ii)	Chymotrypsin	+	+	-	+	0	±
iii)	Carboxypep-tidase	0	-	0	0	0	0
F)	Phagocytosis --						
i)	Elastase	-	+	-	+	0	-
ii)	Collagenase	-	+	-	+	0	-
iii)	Cathepsin B	0	-	0	0	0	0
iv)	Cathepsin C	0	-	0	0	0	0
v)	Cathepsin D	-	-	0	+	0	0
vi)	Cathepsin G	+	+	0	0	0	0
vii)	Mast cell trypsin	-	-	0	0	0	0
viii)	Mast cell chymotrypsin	+	-	0	0	0	0
ix)	Bacterial collagenase	0	+	0	0	0	0
x)	Subtilopeptidase	0	+	0	0	0	0
G)	Metabolism --						
i)	Papain	-	-	0	+	0	0
ii)	Bromelain	-	-	0	+	0	0
iii)	Ficin	-	-	0	+	0	0
H)	Renal function --						
i)	Renin	0	+	0	-	0	0
ii)	Aminopeptidases	0	-	0	0	0	0

Enzyme		Inhibitor					
		α_1-Achy	α_1-PI	AT III	α_2-M	α_2-AP	CI-Inh
I)	Kallikreins --						
i)	Plasma kallikreins	-	+	±	+	±	+
ii)	Urinary	0	-	0	-	0	0
iii)	Saliva	0	-	0	-	0	0
iv)	PKA	0	0	±	-	0	+

* Table from Murano **(1985)**

(+) inhibited;

(±) weakly inhibited;

(-) not inhibited;

(0) data not available.

The half time of association in vivo of some of the proteinases with human plasma proteinase inhibitors are listed in Table III. For an inhibitor to have effective control of a particular enzyme, $t_{½ass}$ should not be more than 10 milliseconds (Travis and Salvesen, 1983). But there are few exceptions e.g. interaction of Cl-Inhibitor with CIr or CIs which probably do not represent true in vivo rates because the enzyme(s) is in a multicomplex. The same is also true for kallikrein and Factor Xa. In addition to this there may be some other undiscovered factors in plasma or tissue that increases the rate of inhibition as heparin activates AT III (Travis and Salvesen, 1983). The physiological role for the inhibitor can be ascertained if an inhibitor is congenitally deficient in an individual with the development of a disease e.g. in case of $alpha_1$-proteinase inhibitor deficiency. But the deficiency states for all the inhibitors have not been reported so far (Travis and Salvesen, 1983).

Thus to assign a physiological function to an inhibitor, we should know -- (i) physical defect associated with its deficiency, (ii) association constant

TABLE III*

HALF-TIME OF ASSOCIATION OF PROTEINASES WITH HUMAN PROTEINASE INHIBITORS[a]

Enzyme	Inhibitor[b]					
	α_1-Pl	α_1-Achy	α_2-M	CI-Inh	α_2-AP	AT-III
Elastase	0.61[c]	-	7.2[c]	-	-	-
Cathepsin G	102[c]	5[c]	93[c]	-	-	-
Trypsin	3,600[c]	-	19[c]	-	617[c]	+
Chymotrypsin	8[c]	27,000[c]	27[c]	-	11,100[c]	-
Kallikrein	+	-	6,125	8,333	+	+
Thrombin	8.3×10^5[c]	-	5×10^5	?	-	847
Plasmin	2.1×10^5[c]	-	+	+	29[c]	+
CIr	-	-	-	2.0×10^5	-	-
CIs	-	-	-	4.7×10^5	-	-
Factor Xa	1.7×10^5	-	3.5×10^5	?	+	2.3×10^5

* Table from Travis and Salvesen (1983)

a In milliseconds

b (-), No interaction; (+), inactivation but no kinetic data; (?), no data available.

c Measured at room temperature

of interaction of inhibitor with enzyme and (iii) the partitioning of the enzyme in question among all plasma proteins. If the deficiency state is not known, the last two sets of data will still help in determining whether the inhibition of an enzyme by an inhibitor is rapid enough to be of physiological significance or not (Travis and Salvesen, 1983).

Alpha$_1$-Antichymotrypsin:

Alpha$_1$-antichymotrypsin described first by Schultze et al., (1962a) was isolated from human serum and partially characterized without knowing its function (Schultze et al., 1962b). Heimburger and Haupt (1965) demonstrated for the first time that this inhibitor causes inhibition of chymotryptic activity only and had no effect on activities of trypsin and plasmin. On electrophoresis of serum proteins the inhibitor was found to be localized in α fraction, accordingly it was termed as alpha$_1$-antichymotrypsin.

Alpha$_1$-antichymotrypsin is mainly synthesized in hepatocytes (Knowles et al., 1980) and the normal level of the inhibitor in plasma/serum ranges from 25 to 60 mg/100 ml(Heimburger, 1975; Travis and Salvesen, 1983). The

inhibitor level shows dependence on age; lower values were found in children and the level increased with increase in age (Weeke and Krasilnikoff, 1972). In adults no concentration difference was found due to sex (Ganrot, 1972; Kelly et al., 1978). The other body fluids having alpha$_1$-antichymotrypsin included urine (Bjerrum and Bog-Hansen, 1975), pleural fluid (Laine and Hayem, 1981), aminiotic fluid (Bhat and Pattabiraman, 1980), seminal plasma (Schill, 1976), sol phase of sputum (Ryley and Brogan,1973), milk (Lindberg,1979), gastric juice, bile, cerebrospinal and synovial fluid (Kawaguchi et al., 1983). By specific staining a number of cells were found to contain alpha$_1$-antichymotrypsin (Motoi et al., 1980; Papadimitriou et al., 1980). These cells among others included mast cells, endothelial cells, epithelioid cells and hepatoma cells. Platelets, lymphoid cells such as T & B lymphocytes and myeloid cell line including myelocytes, neutrophils, basophils etc. were found to be devoid of inhibitor. Thus demonstration of alpha$_1$-antichymotrypsin in /on the cells can be used as a marker for the origin of a particular cell line (Berninger,1985).

Alpha$_1$-antichymotrypsin is an early stage acute phase protein (Aronsen et al., 1972) as its plasma

concentration has been found to increase about two to four times of its basal level (Laurell,1972)within eight hours of tissue damage like surgery (Aronsen et al., 1972; Hlorl et al., 1986), burn injuries (Coombes et al., 1979; Daniels et al., 1974; Moody et al., 1985), Crohn's disease, ulcerative colitis (Weeke and Jarnum, 1971;Chambers et al., 1987) and myocardial infarction (Johansson et al., 1972). Thus the inhibitor might have an important role in protection of body tissue from the damage caused by chymotrypsin like enzymes particularly those released during an inflammatory episode. As $alpha_1$-antichymotrypsin interacts most rapidly with cathepsin G which is involved in the hydrolysis of proteoglycan (Roughley and Barrett, 1977), elastase, collagen (Justice et al., 1987), fibronectin (Vartio et al., 1981) and in the conversion of angiotensin I to biologically active fragment (Gaffer et al., 1980; Wintroub et al., 1981; Reilly et al., 1982; Tonnesen et al., 1982), some vital regulatory roles are proposed for this inhibitor. $Alpha_1$-antichymotrypsin was frequently detected in tumour cell nuclei of human stomach ,breast and pancreas adenocarcinoma and hepatoma (Takada et al., 1982; Ordonez and Manning, 1984; Tahara et al., 1984). Takada et al., (1986) have further observed that $alpha_1$-antichymotrypsin was not synthesized by carcinoma cells but was incorporated

from blood circulation. Alpha$_1$-antichymotrypsin was also found to inhibit DNA polymerase a (Tsuda et al., 1986; 1987;Takada et al.,1988) and thus by inhibiting the DNA replication alpha$_1$-antichymotrypsin present in carcinoma cell may play some important role in host defence mechanism against tumour growth. Alpha$_1$-antichymotrypsin also has an immunoenhancing effect on in vivo response to sheep RBCs in mice (Matsumoto et al., 1981; 1982a). On the other hand it was reported to inhibit human natural cytotoxicity (Katunuma et al., 1978; Hudig et al., 1981). Gravagna et al., (1982) reported that alpha$_1$-antichymotrypsin inhibited both natural killing (NK) and antibody dependent cell mediated cytotoxicity (ADCC). This inhibition is resulted through the binding of the inhibitor on the NK and ADCC target cells and thus neutralizing the lytic signal passed between the effector and target.

1. Isolation:

Alpha$_1$-antichymotrypsin has been isolated from human serum or plasma on several occasions. The methods usually included salt fractionation, gel filtration, ion exchange chromatography and affinity chromatography (Travis et al., 1978b: Siddiqui et al., 1980, Katsunuma et al., 1980; Laine & Hayem, 1981; Abdullah et al., 1983; Laine et

al.,1984b; Yi et al., 1987). The ion exchange resins generally used were strong anion exchanger such as QAE - Sephadex (Siddiqui et al., 1980; Abdullah et al., 1983) and weak anion exchanger like DEAE - Sephadex (Katsunuma et al., 1980). To remove albumin Cibacron Blue Sepharose (Travis et al., 1978b) and anti-albumin Sepharose (Laine and Hayem, 1981; Laine et al., 1984b) were used. Thiopropyl-Sepharose and Thiol-Sepharose columns were used for its isolation because $alpha_1$-antichymotrypsin does not bind to either column whereas many other serum proteins do (Laine and Hayem, 1981). By exploiting its property to bind DNA, DNA - cellulose chromatography was also used for its isolation (Katsunuma et al., 1980; Siddiqui et al., 1980; Abdullah et al., 1983; Yi et al., 1987). Anti - $alpha_1$-antichymotrypsin antibody also has been used to prepare affinity gel (Laine and Hayem, 1981; Laine et al., 1984b). The yield obtained by Katsunuma et al.,(1980) and Siddiqui et al.,(1980) was quite low i.e. about 40 mg of inhibitor per litre of serum. Abdullah et al., (1983) modified their previous procedure (Siddiqui et al., 1980) and got a yield of 80 mg of inhibitor per litre of serum which was found to be comparable to that obtained by Travis et al., (1978b). However, the maximum yield of 250-300 mg

of inhibitor per litre of serum was obtained by Laine et al., (1984b).

Chymotrypsin inhibitor was also isolated from other sources such as snake venom(Liu et al.,1983; Ritonja et al., 1983a ; 1983b), marine turtle egg white (Guha and Sinha, 1984), ascaris (Babin et al., 1984) and silkworm hemolymph (Eguchi and Shomoto, 1984) but none of them was found to be similar to that of human $alpha_1$-antichymotrypsin. However, human plasma $alpha_1$-antichymotrypsin shows immunological identity with inhibitor isolated from the plasma of Pan trogiodytes (Chimpanzee) and Erythrocebus pattas (an old world monkey) but not with nine non-primate eutheria plasmas (Bauer, 1973). In porcine serum, no counterpart of human $alpha_1$-antichymotrypsin was found although proteinase inhibitors similar to other human proteinase inhibitors were detected (Westrom, 1979a; 1979b) However, from an invertebrate source i.e. silkworm larval hemolymph chymotrypsin inhibitor was isolated which resembles with human $alpha_1$-antichymotrypsin in size, mode of association with proteinases and active site location (Sasaki,1985).

2. Properties:

i) Molecular Properties

In general alpha$_1$-antichymotrypsin from human blood has been characterized for its molecular weight. It has been determined usually by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Travis et al., 1978b; Siddiqui et al., 1980; Laine and Hayem, 1981; Laine et al., 1984b) and on two occasions by sedimentation equilibrium (Travis et al., 1978b; Katsunuma et al., 1980). The values of molecular weight obtained, under native and denaturing conditions, thus far are listed in Table IV. The molecular weight of alpha$_1$-antichymotrypsin determined both by sodium dodecyl sulphate polyacrylamide gel electrophoresis and sedimentation equilibrium was found to be same i.e. 66 kDa. Since the inhibitor contains 26% carbohydrate (Tsuda et al., 1982) the molecular weight of the polypeptide would be about 47 kDa (Matsumoto et al., 1982b). From amino acid sequence the molecular weight of the inhibitor was calculated as 46 kDa (Chandra et al., 1983).

As the molecular weight of the inhibitor was found to be same with or without 2-mercaptoethanol. It was concluded that inhibitor consists of a single polypeptide chain (Travis et al., 1978b; Siddiqui et al., 1980; Laine

TABLE IV

MOLECULAR WEIGHT OF HUMAN ALPHA$_1$ - ANTICHYMOTRYPSIN

	Condition/system	Method	Molecular weight (kDa)	Reference
i)	<u>under native condition</u> -			
	a) in 50 mM Tris-HCl buffer,pH 8.0, containing 0.05 M sodium chloride.	sedimentation equilibrium	65	Travis et al., (1978b)
	b) in 10 mM Tris-HCl buffer, pH 7.8, containing 0.1 M sodium chloride	"	64	Katsunuma et al. (1980)
ii)	<u>under denaturing condition</u> -			
	a)in 1% SDS (under reducing and nonreducing conditions)	SDS-PAGE	58	Laine and Hayem (1981) Laine et al., (1984b)
	b) in 0.1% SDS (under reducing and nonreducing conditions)	"	65-68	Travis et al., (1978b) Siddiqui et al., (1980)

and Hayem, 1981). Under native conditions the gel filtration data on Sephadex G-200 column also indicates its monomeric nature (Siddiqui et al., 1980).

The sedimentation coefficient of the inhibitor was determined as 3.9 s (Schwick and Haupt, 1984). The partial specific volume of the inhibitor, calculated from its amino acid and carbohydrate composition, was found to be 0.715 ml/g (Travis et al.,1978b). The inhibitor preparation which was homogeneous with respect to size as indicated by SDS-PAGE gave as many as seven protein bands on isoelectric focussing with isoelectric points lying in the range of 4.1-4.45 (Gianazza and Arnaud, 1981). This microheterogeneity is not entirely due to unequal sialylation of the protein since microheterogeneity persists even after neuraminidase treatment.

The human $alpha_1$-antichymotrypsin stored for one month in 0.05 M Tris, 0.05 M sodium chloride, pH 8.0, at $4^{\circ}C$ retained its inhibitory activity; under frozen conditions the inhibitor can be kept without inactivation for substantially longer time (Travis et al., 1978b). The inhibitor was found to be stable in the pH range of 5.0-8.0, however, its acidification to pH 3.0 abolished its activity

(Travis et al., 1978a). When the concentration of inhibitor is increased to more than 3 mg/ml, the inhibitor undergoes polymerization and eventually precipitation (Travis et al.,1978b). The inhibitor is heat stable upto 50°C beyond which further heating causes its inactivation (Matsumoto et al., 1982b; Tsuda et al., 1986). Specific oxidants of methionine residue like N-bromosuccinimide or N-chlorosuccinimide (Travis et al., 1978b) and myeloperoxide in the presence of H_2O_2 and chloride ion (Matheson et al., 1979) fail to inactivate alpha$_1$-antichymotrypsin. However, on exposure of inhibitor to ozone, 50% of its inhibitory activity was lost and amino acid analysis of ozone exposed inhibitor showed the destruction of Trp, Met, Tyr and His (Smith et al., 1987).

ii) Optical properties

Human alpha$_1$-antichymotrypsin has been found to absorb at 280 nm and using 1% solution of the inhibitor its extinction coefficient was determined as 6.2 cm^2g^{-1} (Travis et al., 1978b). Laine et al., (1982b) have studied the circular dichroism spectra of human alpha$_1$-antichymotrypsin in 10 mM sodium phosphate buffer, pH 7.5, containing 0.3 M sodium chloride and 0.2% sodium azide in the wavelength

region of 200-350 nm. The characteristic features of the near UV CD-spectra of the inhibitor were peak at 267 nm, shoulders at 262 and 272 nm, a negative band at 284 nm, a positive band at 298 nm and the isobestic points at 277 and 292 nm. The fine structure between 260-275 nm were assigned to phenylalanine residues (Strickland, 1974). The trough at 284 nm was due to tyrosine and tryptophan residues whereas the positive band at 298 nm was probably due to tryptophan residues. The fine structure of the CD-spectra of $alpha_1$-antichymotrypsin in the wavelength region of 260-350 nm were significantly altered upon its interaction with specific enzymes like cathepsin G and chymotrypsin. However, the far UV CD-spectra of the inhibitor showing minima at 222 nm and a shoulder at 214 nm remain uninfluenced by its interaction with the proteolytic enzymes. It should be pointed out that for a typical helical polypeptide the characteristic feature of the CD-spectra include minima at 208 and 222 nm (Greenfield and Fasman, 1969). In the CD spectra of the inhibitor the trough at 208 nm is replaced by a shoulder at 214 nm which might be due to the presence of slightly deformed alpha-helix.

iii) Chemical properties

a) Amino acid and carbohydrate composition

Human alpha$_1$-antichymotrypsin contains 408 amino acid residues in a single chain with covalently bound carbohydrate moieties (Chandra et al., 1983). The amino acid composition of the inhibitor has been determined by chemical methods (Heimburger et al., 1971; Travis et al., 1978b; Laine and Hayem, 1981) and has also been deduced by base sequence of cDNA for alpha$_1$-antichymotrypsin (Chandra et al., 1983). The results are summarized in Table V. It can be seen that number of basic amino acid residues i.e. Lys, Arg and His are 52 and acidic amino acid residues are also 52 but there are 14 sialic acid residues in the inhibitor. Thus this composition is consistent with the acidic behaviour of the inhibitor. The total number of hydrophobic amino acid residues are 179 which form about 44% of the total amino acids. The number of Trp, Tyr and Phe residues are respectively 4, 10 and 24. For every 24 amino acid residues, there is one proline residue in the molecule. The carbohydrate moieties present in the inhibitor are 36-41 neutral hexose residues, 25-35 N-acetyl hexosamine and 11-16 sialic acid residues per mole of inhibitor. Heimburger et al., (1971) reported that neutral

TABLE V

AMINO ACID AND CARBOHYDRATE COMPOSITION OF HUMAN ALPHA$_1$-ANTICHYMOTRYPSIN

Amino acid/carbohydrate residues	residues/ mole			
	A	B	C	D
Lysine	28	27	28	27
Histidine	9	9	9	8
Arginine	15	15	18	17
Aspartic acid/Asparagine	47	49	48	26/17
Glutamic acid/Glutamine	54	50	50	26/18
Threonine	32	31	30	27
Serine	31	32	34	37
Proline	15	15	18	16
Glycine	16	18	20	15
Alanine	31	34	33	30
½-cystine	--	2	--	2
Valine	25	27	21	24
Methionine	12	11	10	12
Isoleucine	20	23	15	21
Leucine	54	56	53	48
Tyrosine	10	9	11	9
Phenylalanine	26	24	21	24
Tryptophan	3	4	ND	4
Neutral sugar	41	38-40	35	ND
N-Acetyl hexosamine	25	30-35	25	ND
Sialic acid	14	11-14	10	ND

References: A) Heimburger et al.,(1971);
B) Travis et al., (1978b);
C) Calculated according to the data of Laine and Hayem (1981);
D) Chandra et al., (1983).

ND - not determined

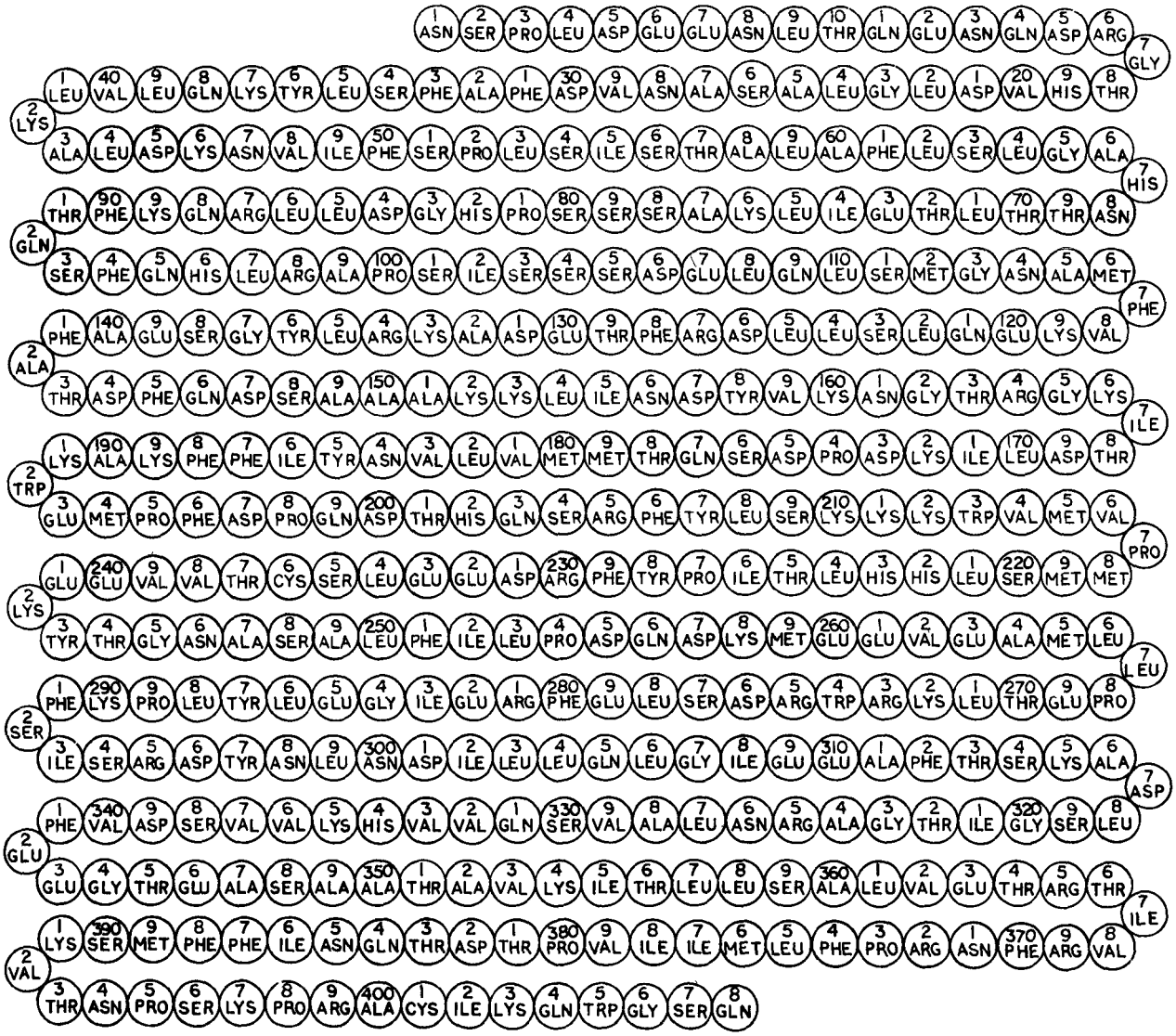
hexose moieties present are galctose and mannose. Fucose (3%) is also present and no N-acetyl galactosamine was found in alpha$_1$-antichymotrypsin.

b) Amino acid sequence

The amino acid sequence of the human alpha$_1$-antichymotrypsin as deduced from cDNA (Chandra et al.,1983) is given in Fig. 1. Being a secretory protein, the inhibitor contains a signal peptide of 22-25 amino acid residues in which majority of the amino acid residues are hydrophobic in nature. The N-terminal amino acid residue is asparagine which was also determined by chemical method (Laine et al., 1984b). This is in contrast to the earlier report of arginine as the N-terminal residue (Travis et al., 1978b; Laine and Hayem, 1981). The C-terminal amino acid residue is glutamine which differs from earlier finding where glycine was found to be C-terminal residue of the inhibitor (Travis et al., 1978b).

The distribution of acidic and basic amino acid residues in human alpha$_1$-antichymotrypsin, as derived from amino acid sequence given in Fig.1, are graphically shown in Fig. 2. It can be seen that majority of the side chain carboxyl groups are found in the regions in sequence namely

Fig.1. Amino acid sequence of $alpha_1$-antichymotrypsin as obtained by cDNA studies (Chandra et al., 1983).



AMINO ACID SEQUENCE OF HUMAN ALPHA$_1$ - ANTICHYMOTRYPSIN

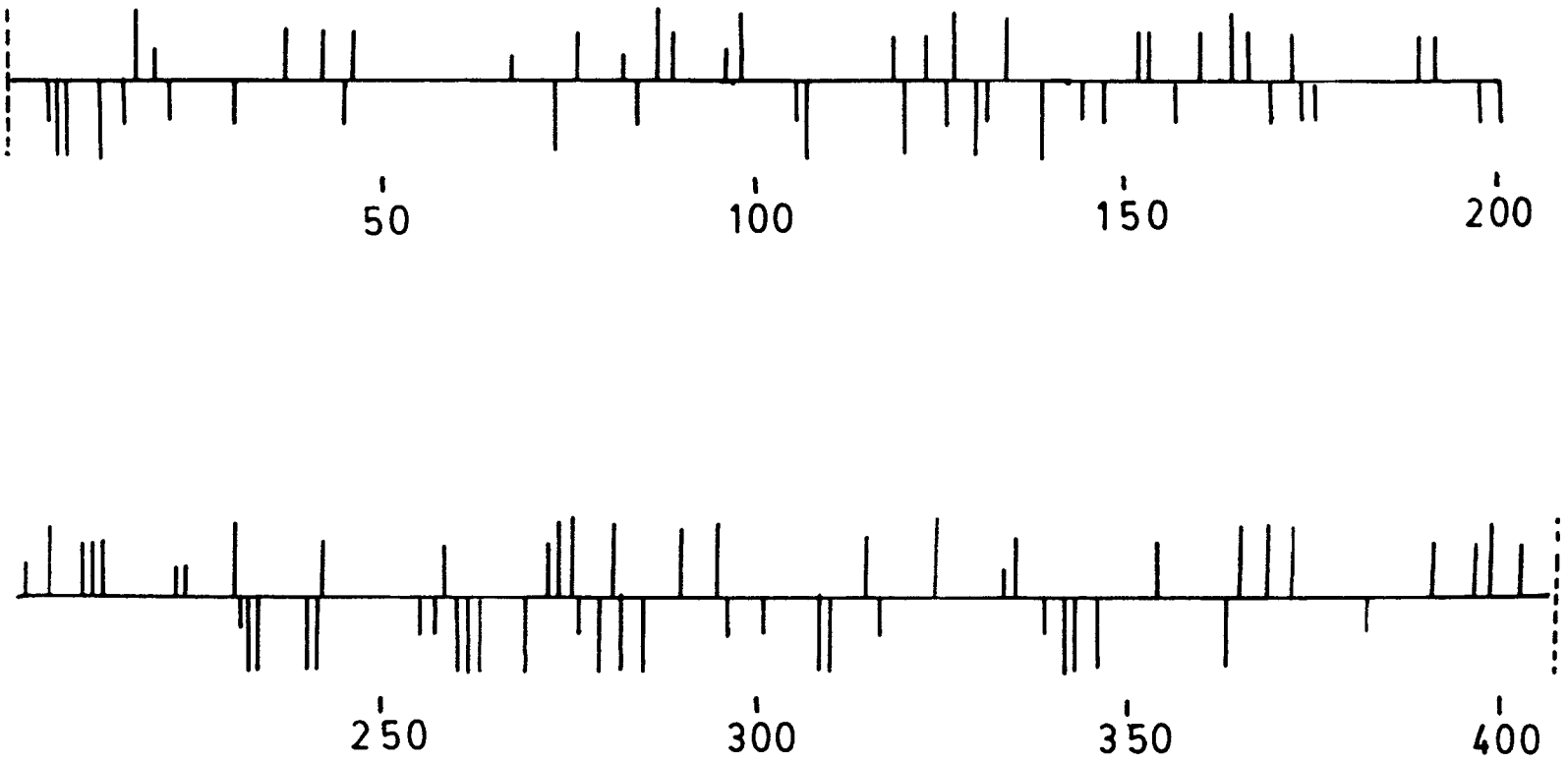


Fig. 2. Distribution of arginine (⊥), lysine (⊥), histidine (⊥), aspartic acid (⊤) and glutamic acid (⊤) in human alpha$_1$- antichymotrypsin.

Asp 5 - Asp 30, Glu 120 - Asp 147 and Asp 255 - Glu 285. There are three clusters of positively charged groups (i.e. basic amino acid residues) located between His 65 - Arg 98, Lys 152 - Lys 172, and Lys 272 - Arg 295. Out of the 30 amino acid residues in the N-terminal portion 7 are acidic amino acid residues and only two are basic. Accordingly out of 28 amino acid residues at the C-terminal end 4 are basic amino acid residues and one is acidic. Thus N and C terminal portions of the inhibitor are predominantly acidic and basic in nature. The distribution of hydrophobic and hydrophilic amino acid residues appears to be random as can be seen in Fig.3.

Reactive site sequence

The lone reactive site of the inhibitor for the corresponding proteolytic enzymes lies near the carboxyl end. The reactive site amino acid residues have been identified as Leu — Ser (Morii and Travis, 1983) and located at positions 358 and 359 of the inhibitor (Chandra et al., 1983). Other functionally relevant residues of the inhibitor are given in Table VI which also contains the active site residues of other serine proteinase inhibitors. From the table it is clear that inhibitory specificity of a

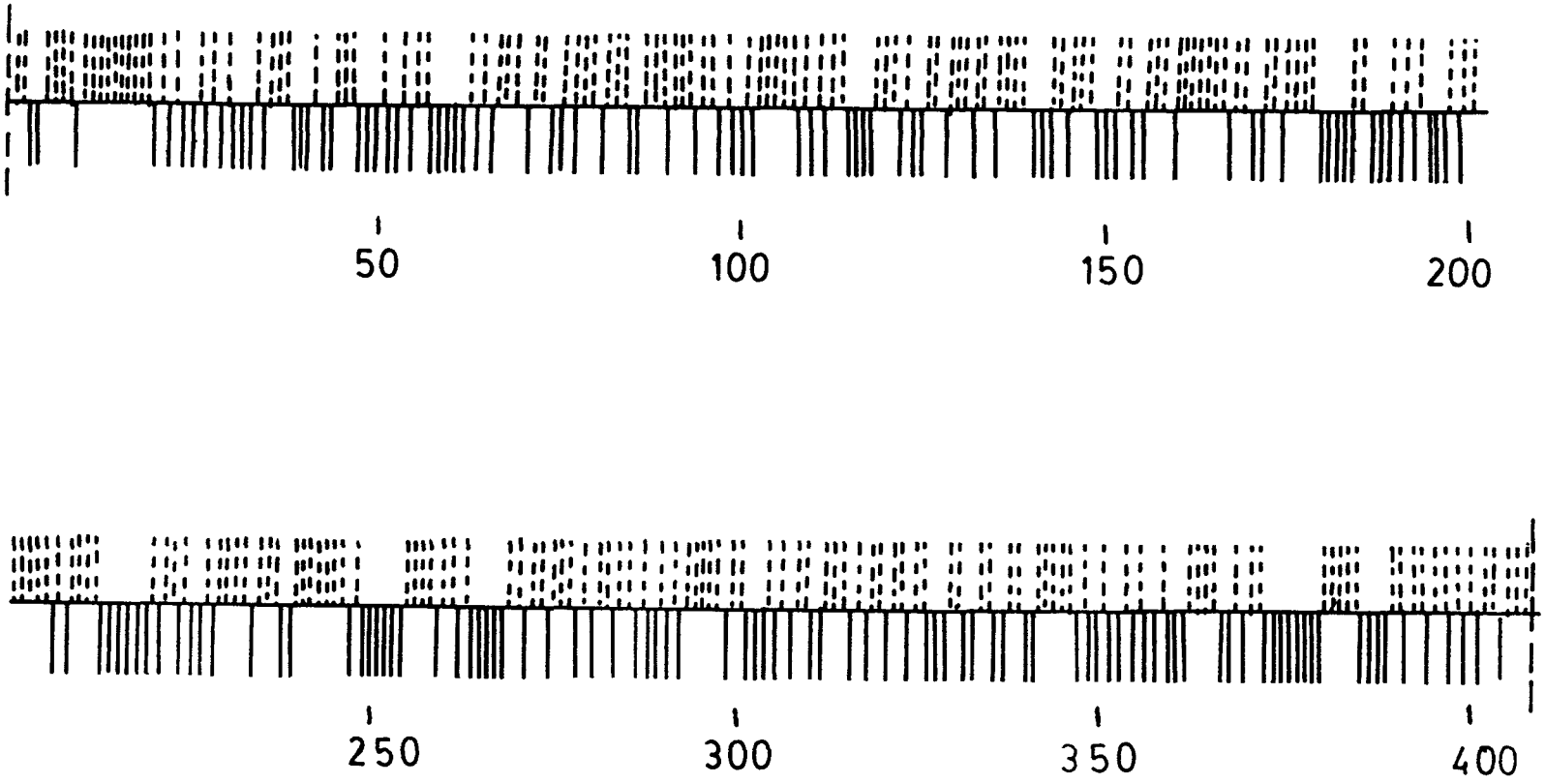


Fig. 3. Distribution of hydrophilic (—|—) and hydrophobic (—┬—) amino acid residues in human alpha$_1$-antichymotrypsin.

TABLE VI

AMINO ACID SEQUENCE OF REACTIVE CENTRES OF SERINE PROTEINASE INHIBITORS

	Serine proteinase inhibitor	Target enzyme	Reactive centre residues					
			P_2	P_1	P'_1	P'_2	P'_3	P'_4
1.	$Alpha_1$-antichymotrypsin	chymase	Leu	Leu	Ser	Ala	Leu	Val
2.	$Alpha_1$-antitrypsin	elastase	Pro	Met	Ser	Ile	Pro	Pro
3.	Valine mutant	elastase	Pro	Val	Ser	Ile	Pro	Pro
4.	Pitsburgh mutant	thrombin	Pro	Arg	Ser	Ile	Pro	Pro
5.	Antithrombin III	thrombin	Gly	Arg	Ser	Leu	Asn	Pro
6.	Mouse contrapsin	trypsin	Arg	Lys	Ala	Ile	Leu	Pro
7.	C I - inhibitor	kallikreins	Ala	Arg	Thr	Leu	Leu	Val

Table from Carrell and Travis (1985)

serine proteinase inhibitor is primarily defined by a single amino acid residue at the reactive centre i.e. P_1 residue. For example, methionine (or valine) for elastase, lysine for trypsin, leucine for chymotrypsin and arginine for thrombin. P'_1 position appears to be invariant in five of the seven inhibitors listed in Table VI. Semisynthetic substitution studies showed that serine in this position is better than any other amino acid residue whose substitution has been attempted (Laskowaski and Kato, 1980). While alanine and threonine can substitute for serine at P'_1 position as in mouse contrapsin (Hill et al., 1984) and CI-inhibitor, the replacement of serine by proline renders the inhibitor inactive towards the proteases (Laskowaski and Kato, 1980).

Mutation at P_1 position alters the specificity of the inhibitor towards proteolytic enzyme e.g. mutation of 358 Met —> Arg in $alpha_1$-antitrypsin Pitsburgh mutant results in the change of specificity of the inhibitor from trypsin to thrombin (Owen et al., 1983). The functional role played by other residues i.e. P_2, P_3, P_4, P'_2, P'_3, P'_4 etc. are yet to be studied. However, in case of α_1-AT Christchurch, 363 Glu —> Lys mutation at P'_5 position does

not effect its inhibitory activity indicating that P'_5 residue is not critically involved in determining the specificity or mechanism of action of inhibitor (Brennan and Carrell, 1986). With the exception of mouse contrapsin, in all serine proteinase inhibitors the residue at position P_2 is neutral, consistent with its burial in the enzyme inhibitor interface. However, at positions P_3, P_2,P'_3 and P'_4 proline helps in maintaining the reactive site geometry even though this can be achieved without proline (Laskowaski and Kato, 1980). Removal of P'_1 amino acid residue or insertion of an additional residue between P_1 and P'_1 in soyabean trypsin inhibitor abolish its inhibitory activity. According to Kowalski and Laskowaski (1976a; 1976b) this may be due to the fact that either reformation of P_1 - P'_2 peptide bond is not possible or the required site geometry is destroyed.

3. **Limited proteolysis:**

$Alpha_1$-antichymotrypsin and $alpha_1$-proteinase inhibitor on incubation with catalytic amount of snake venom or bacterial metalloproteinases are converted into inactive modified form while other serine proteinase inhibitors like CI-Inh, antithrombin III and $alpha_2$-

antiplasmin first converted into active modified form which on further cleavage resulted in the formation of inactive modified inhibitor (Kress, 1986). Amino acid sequence analysis showed that noninactivating cleavage occurred in the N-terminal region of the inhibitor while in all cases inactivation resulted from the cleavage of a single bond near, but not at, the active site in the C-terminal region of the inhibitor. Thus there are two regions in the inhibitors which are susceptible to limited proteolysis, one near the N-terminal end and other in the C-terminal end at exposed reactive site of the inhibitor.

4. **Sequence homology:**

Human plasma serine proteinase inhibitors formed a family called "Serpins". These serpins share about 30% of common sequence which rises to 70% when only hydrophobic amino acid residues are considered (Carrell and Travis, 1985). Since the hydrophobic amino acid residues form interior of the native protein conformation, their conservation during evolution is quite expected.

Comparison of amino acid sequences of three serine proteinase inhibitors i.e. $alpha_1$-antichymotrypsin, $alpha_1$-antitrypsin and antithrombin III revealed 33-42%

homology (Chandra et al., 1983). Dot matrix analysis indicated higher level of sequence homology throughout the amino acid sequences of alpha$_1$-antichymotrypsin and alpha$_1$-antitrypsin. However, as compared to C-terminal half, the sequence at N-terminal half of two proteinase inhibitors exhibited significantly higher level of homology. The comparison of alpha$_1$-antichymotrypsin with antithrombin III showed a much more scattered pattern on dot matrix analysis indicating that the two sequences are much less conserved. The amino acid sequences surrounding the active sites of these three proteinase inhibitors were also compared (See Fig. 4) and it was found that homology at the active sites is much less extensive than 5-10 residues away from the active sites.

The gene for alpha$_1$-antichymotrypsin is located in the q31 - q32.3 region of human chromosome 14 (Rabin et al., 1986) and is approximately 12 kb long containing five exons and four introns as shown in Fig.5 (Bao et al., 1987). All five exon containing regions of the gene are sequenced and the sequences at each exon-intron boundary is shown in Fig.6. The gene for alpha$_1$-antitrypsin is also located at the q31-q32 region of human chromosome 14 (Schroeder et

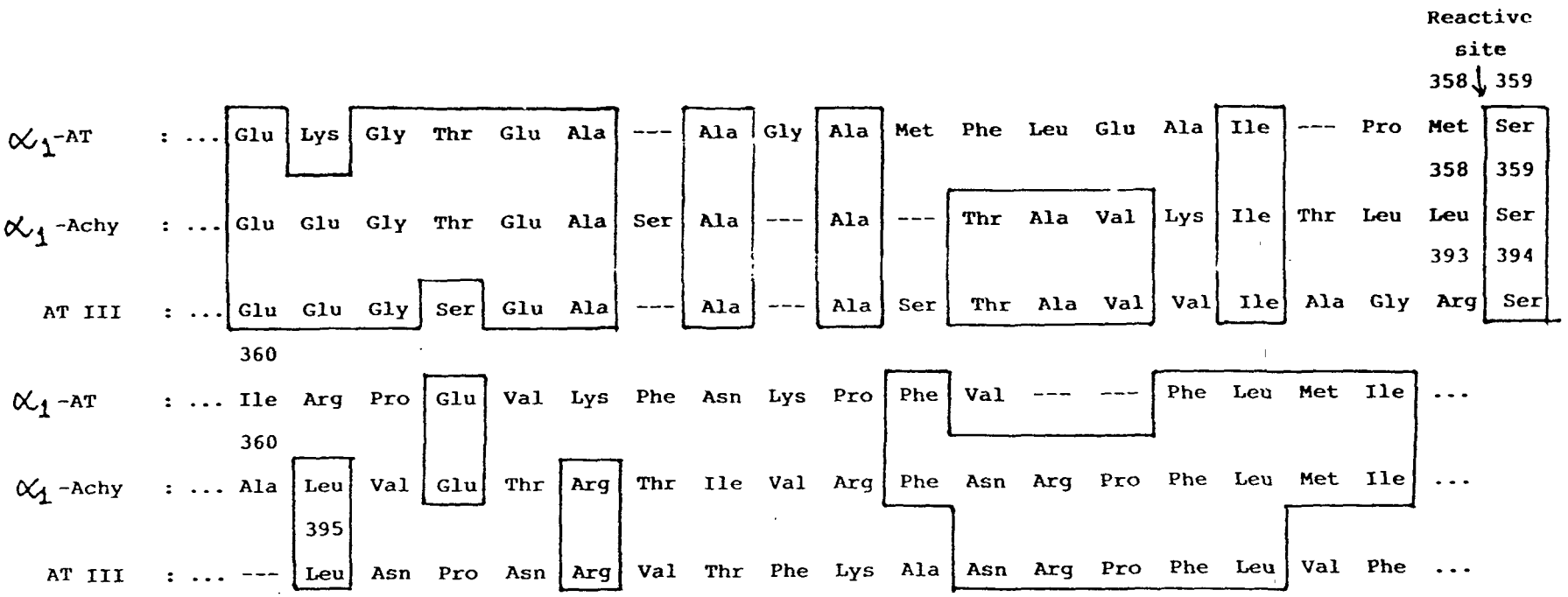


Fig.4. Comparison of active site sequences of α_1-antichymotrypsin (α_1-Achy), α_1-antitrypsin (α_1-AT) and antithrombin III (AT III). The number refers to the amino acid residues of the mature proteins (Chandra et al., 1983).

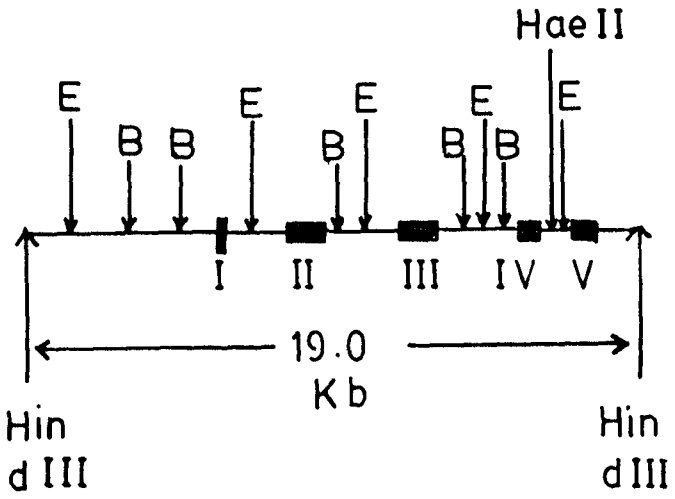


Fig. 5. Restriction map of Hin d III, Eco RI (E), Bam HI (B) and Hae II sites in human chromosomal alpha$_1$-antichymotrypsin gene. Exons are denoted by solid boxes and marked I-V (Bao et al., 1987).

```
        Exon I                Intron A          Exon II

..... TCC CTG AGG CAG geta atc.....dtca cag TTG AGA ATG GAG.....
                                                 Met Glu
                                                 -25 -24

        Exon II               Intron B          Exon III

..... TTC TTT AAA G    gtg agt......ctt cag  CC AAA TGG GAG.....
      Phe Phe Lys                            Ala Lys Trp Glu.....
      187 188 189                            190 191 192 193

        Exon III              Intron C          Exon IV

..... CTG GAG TTC AG   gtg att......ttc tag  A GAG ATA GGT .....
      Leu Glu Phe Arg                          Glu Ile Gly
      278 279 280 281                          282 283 284

        Exon IV               Intron D          Exon V

..... GCA GTC TCC CAG  gtg agt..... gac gag GTG GTC CAT AAG.....
      Ala Val Ser Gln                        Val Val His Lys
      328 329 330 331                        332 333 334 335
```

Fig.6. Exon - intron junction sequences of alpha$_1$-antichymotrypsin gene (Bao et al., 1987).

Exon sequences are in capital letters and intron sequences in small letters. The numbers shown denote the positions of corresponding amino acids in the gene.

et al., 1985; Rabin et al., 1986) and in both the genes the number and positions of introns and type of exon intron junctions are similar, if not identical (Bao et al., 1987). The similar organization of alpha$_1$-antichymotrypsin and alpha$_1$-antitrypsin gene is consistent with the high degree of homology in their amino acid sequences. Despite the fact that there is sequence homology in alpha$_1$-antichymotrypsin and antithrombin III the number and positions of introns in two genes are very much different (Bao et al., 1987).

5. Interaction of alpha$_1$-antichymotrypsin with serine proteinases:

Human alpha$_1$-antichymotrypsin is specific for chymotrypsin, both bovine and human (Travis et al., 1978a; 1978b; Laine et al., 1984a), leukocyte cathepsin G (Laine et al., 1982a), cathepsin G from sputum (Laine et al., 1984b), dog and human mast cell chymases (Travis et al., 1978a; Reilly et al., 1982), human pancreatic elastase 2 (Laine et al., 1985; Davril et al., 1987) and porcine pancreatic elastase (Laine et al., 1985). The values of the rate constant of interaction of inhibitor with some serine proteinases is listed in Table VII. Evidently human

TABLE VII

ASSOCIATION RATE CONSTANTS FOR THE INTERACTION OF $ALPHA_1$ - ANTICHYMOTRYPSIN WITH DIFFERENT ENZYMES

	Enzymes	Association rate constant ($M^{-1}s^{-1}$)	References
1.	Human cathepsin G	$5.1 \pm 0.7 \times 10^7$	Beatty et al., (1980)
2.	Human chymotrypsin	$1.0 \pm 0.1 \times 10^4$	Beatty et al., (1980)
3.	Bovine chymotrypsin	$6.0 \pm 1.0 \times 10^4$	Beatty et al., (1980)
4.	Human pancreatic elastase 2	$8.9 \pm 1.3 \times 10^5$	Laine et al., (1985);Davril et al.,(1987)

$alpha_1$-antichymotrypsin associates with human cathepsin G at the fastest rate followed by human pancreatic elastase 2. Bovine chymotrypsin was indistinguishable from human chymotrypsin The fact that with human cathepsin G the rate of complex formation is fastest suggests that the inhibitor preferentially interactswith cathepsin G (Travis et al., 1978a). It was also found to inhibit porcine pancreatic elastase but the inhibition is so slow that it has no physiological significance (Laine et al., 1985). Neither human trypsin nor neutrophil elastase was found to be inhibited by $alpha_1$-antichymotrypsin (Travis et al., 1978a).

It is also possible to calculate half time of inhibition, $t_{\frac{1}{2}}$, from the kinetic data of association of inhibitor with enzyme (Beith, 1980). The values for $t_{\frac{1}{2}}$ for cathepsin G and chymotrypsin was calculated as 5 milliseconds and 27 seconds respectively (Travis and Salvesen, 1983).

Mechanism of interaction of inhibitor with enzyme

The proteinase inhibitor interacts with the corresponding proteinases through the binding of active site of proteinase to the corresponding substrate like

region on the inhibitor i.e. reactive site of inhibitor (Ozawa and Laskowaski,1960). The reactive site of the inhibitor is fairly rigid, consequently little or no conformational changes occur when inhibitor combines with enzyme (Laskowaski and Kato,1980). The peptide bond joining P_1 and P'_1 is hydrolyzed during the complex formation (Travis and Salvesen, 1983). The value of k_{cat}/K_m for the hydrolysis of this peptide bond at neutral pH is very high, 10^4-10^6 M^{-1} s^{-1} (Estell et al., 1980) as compared to the value for normal substrate i.e. about 10^3 M^{-1} s^{-1} (Laskowaski and Kato, 1980). However the individual values of k_{cat} and K_m for inhibitor are several orders of magnitudes lower than those for normal substrate (Laskowaski and Kato, 1980). Thus the hydrolysis of the peptide bond (P_1 - P'_1) is very slow and does not proceed to completion (Laskowaski and Kato, 1980). The rate of complex formation of enzyme with modified inhibitor i.e. inhibitor with peptide bond cleaved is much lower than that from virgin inhibitor and in few cases this difference is so large that it led to the assumption that the modified inhibitor was inactive, however, on longer incubation it was found to be an error (Laskowaski and Kato,1980).

According to Travis and Salvesen (1983) the enzyme and inhibitor interaction takes place according to following scheme:-

$$E + I \rightleftharpoons EI \rightleftharpoons C \rightleftharpoons EI^* \rightleftharpoons E + I^* \quad (2)$$

Where E and I refer to proteinase and inhibitor, respectively, I* is the modified inhibitor and C is the stable complex. In the stable complex C the scissile peptide bond (P_1 - P'_1) of the inhibitor is not cleaved. But the carbonyl carbon of reactive site peptide bond (P_1 residue) is distorted about halfway towards tetrahedral state from its fully trigonal state and it was believed that this distortion is caused by O^{γ} of catalytic Ser^{195} of enzyme (Laskowaski and Kato, 1980). However, this view was questioned as it was found that anhydrotrypsin, where the catalytic Ser^{195} is dehydrated to dehydrolanyl^{195}, can form stable complexes with inhibitor of comparable strength (Ako et al., 1974), having the same geometry (Huber et al., 1975) and almost with same tetrahedral dstortion as in a complex with trypsin (Huber et al., 1974). Accordingly

Kraut (1977) proposed that this partial tetrahedral distortion is the result of attraction between the carbonyl oxygen of the inhibitor and the oxyanion hole of the enzyme, the NH's of glycine^{193} and serine^{195}. Thus the carbonyl carbon is now ready for the attack by oxygen O^{γ} of serine^{195}, but in the stable complex the attack has not yet taken place (Laskowaski and Kato, 1980). This bond in addition to other interactions including hydrogen bonds, Van der Waal's interactions, hydrogen bonds and salt bridges in the close contact area between the reactive site of inhibitor and enzyme, all contribute to the characteristic stability of the complex (Laskowaski and Kato,1980).

Alpha$_1$-antichymotrypsin and other serine proteinase inhibitors obey atleast a part of the standard mechanism i.e. they rapidly from complexes with their target enzymes via reactive site region. However, the modified inhibitor formed by cleavage of reactive site peptide bond is inactive and cannot recombine with proteinases e.g. cathepsin G and chymotrypsin on incubation with alpha$_1$-antichymotrypsin produce an inactive form of inhibitor (Laine et al., 1982a). The rate of breakdown of complexes

to form inactive inhibitor is not reported in case of $alpha_1$-antichymotrypsin - proteinase complex but for various proteinase - $alpha_1$-proteinase inhibitor complex, it was found to be quite slow i.e. 6×10^6 sec^{-1} - 3×10^8 sec^{-1} (Aubry and Beith, 1972; Beatty et al., 1982). The following kinetic scheme was summarized for interaction of $alpha_1$-antichymotrypsin with enzyme (chymotrypsin) by Laine et al., (1984a) ---

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* + \text{peptide} \qquad (3)$$

$$EI^* \xrightarrow{k_3} E + I^*$$

where E is enzyme, I the inhibitor, EI an enzyme substrate type complex, EI* the covalent complex and I* the modofied

inhibitor. The k_{-1} path was reported in case of alpha$_1$-proteinase inhibitor by Beatty et al., (1982). However, in case of alpha$_1$-antichymotrypsin this path has not yet been established. It was also found that the complex (alpha$_1$-proteinase inhibitor - chymotrypsin) does not dissociate if the complex had been previously treated with diisopropyl fluorophosphate (Lobermann et al., 1982). Therefore, it is still unclear that whether the breakdown of the complex is due to the proteolytic activity of the released enzyme or there is inherent instability of the complex. The inactive modified alpha$_1$-antichymotrypsin can react with antibodies raised against native inhibitor indicating that it must have retained the antigenic determinants of native inhibitor (Laine et al., 1984a).

Alpha$_2$-macroglobulin plays an important secondary role in backing up the primary function of the inhibitor. In vitro, the experiments showed that all the proteinases bound to serine proteinase inhibitor dissociate, although slowly, and transferred to alpha$_2$-macroglobulin. The complex formed with alpha$_2$-macroglobulin is irreversible and does not dissociate (Laskowaski and Kato,1980). In vivo, this effect is greatly exaggerated since

alpha$_2$-macroglobulin - proteinase complexes are cleared very rapidly by reticuloendothelial cells (Debanne et al., 1975; Ohlsson and Laurell, 1976), the half life is about 10 minutes in man (Balldin et al., 1978). Thus essentially all the proteinases are cleared by α_2M - pathway and other serine proteinase inhibitors are only transiently involved in transferring the proteinases to be cleared. But in mouse it was found that clearance of α_2-M-complex is separate from that of other inhibitor complexes (Fretz and Gan, 1980; Fuchs et al., 1982). Therefore, the physiological significance of this mechanism remains equivocal.

A review of the existing literature shows that except human alpha$_1$-antichymotrypsin, inhibitor of identical specificity from other mammalian blood have not been investigated in essential detail thus far. In fact no such inhibitor could be detected in porcine serum (Westrom, 1979a; 1979b). Strikingly chymotrypsin inhibitor from some invertebrates have been found to vary considerably in molecular properties (Liu et al., 1983; Ritonja et al., 1983a; 1983b; Guha and Sinha, 1984; Babin et al., 1984). In this thesis we report the isolation and characterization

of chymotrypsin inhibitor from goat plasma which has not been studied thus far. The properties of plasma and serum goat chymotrypsin inhibitors were found to be indistinguishable. Some molecular and functional properties of goat chymotrypsin inhibitor are being presented here for the first time.

EXPERIMENTAL

A. Materials:

1. Proteins

Bovine serum albumin (lot no. 100F - 033), ovalbumin (lot No. 105C - 8022), pepsin (lot No. 60F - 8056), chymotrypsinogen - A (lot no. 40F - 8050), cytochrome c (lot no.09C - 088), trypsin (lot no. 103F - 8075) and bovine pancreatic chymotrypsin (lot no. C - 4129) were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Casein was isolated from skimmed milk by acid precipitation followed by washing with alcohol and ether as described earlier (Maheshwari , 1979).

2. Buffer components

The monobasic and dibasic salts of phosphoric acid and sodium chloride for the preparation of sodium phosphate buffer were from Sarabhai M. Chemicals, Baroda, India. Potassium hydrogen phthalate and sodium tetraborate which were used for the preparation of standard buffers, were obtained from BDH, Bombay, India. For the preparation of Tris-glycine buffer, Tris (lot no.T-1378)

was purchased from Sigma Chemical Company, St.Louis, Mo., USA and glycine from BDH, Bombay, India.

3. Column chromatographic media

DEAE - cellulose (lot no. 43C - 1140), Dowex 1x4 - 100 (lot no. 39C - 0273) and Dowex 50x4 - 100 (lot no. 43C - 1140), were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

4. Reagents used for gel electrophoresis

Reagents used in electrophoresis with their sources in parenthesis were: acrylamide (E.Merck, Dramstadt, Germany), N,N' - methylene-bis-acrylamide (Reanal, Budapest, Hungary),N,N,N',N' - tetramethylethylenediamine (Ferak, Berlin, West Germany), ammonium persulphate (E. Merck, Dramstadt, Germany), riboflavin and dichlorodimethyl silane (BDH, Poole, England), sodium dodecyl sulphate (BDH, Bombay, India), 2-mercaptoethanol (BDH, Poole, England) , coomassie brilliant blue R (lot no. B - 0630, Sigma Chemical Company, St. Louis, Mo., USA), glycerol, sucrose, isopropanol and chloroform (BDH, Bombay, India).

5. Miscellaneous reagents

Other reagents used were: N-acetylneuraminic acid (lot no. A - 2501),pCMB (lot no. T-7254) from Sigma Chemical Company, St. Louis, Mo., USA, agar (S.D. Fine Chemicals, Bombay, India), phenol, sodium metaperiodate, sodium arsenite, thiobarbituric acid, sodium sulphate and cyclohexanone (BDH, Bombay, India). The rest of the chemicals used were of analytical grade.

All glass distilled water was used throughout the experiments.

B. **Methods:**

1. Preparation of solutions

i) Preparation of Folin-phenol reagent

The Folin - phenol reagent was prepared by the method of Folin and Ciocalteu (1927). Hundred grams of sodium tungstate, 25 grams of sodium molybdate, 700 ml of distilled water, 48.2 ml of orthophosphoric acid and 100 ml of concentrated hydrochloric acid were mixed together and the contents were refluxed gently for 10 hours in a round bottom flask wrapped with black carbon paper.

After cooling, 128.88 grams of lithium sulphate, 50 ml of distilled water and few drops of bromine were added. The mixture was boiled for 15 minutes to remove excess bromine. The solution was cooled, filtered and diluted to one litre. The reagent was stored in a brown coloured bottle and it was diluted five times with distilled water before use.

ii) Preparation of copper reagent

The copper reagent was prepared by mixing 4% sodium carbonate, 4% sodium potassium tartarate and 2% copper sulphate in a ratio of 100 : 1 : 1.

iii) Preparation of Schiff reagent

The Schiff reagent was prepared by dissolving 1.25 grams of basic fuchsin in 250 ml of distilled water, then adding 5 grams of sodium metabisulphite and 50 ml 1N HCl. The solution was stirred for 6 hours at room temperature and then decolourised with activated charcoal.

iv) Preparation of pHMB solution

p-chloromercuribenzoate was dissolved in alkaline aqueous solution in order to convert it into pHMB. This solution was prepared in 10 mM sodium phosphate buffer, pH 7.0, and its

concentration was measured spectrophotometrically by measuring optical density at 232 nm using molar extinction coefficient of 16,900 (Scoffone and Fontana ,1970).

v) Preparation of deionized protein solution

The protein solution was dialyzed extensively against deionized water which was previously prepared by passing glass distilled water through a mixed ion exchange resin column prepared from Dowex 1x4 - 100 and Dowex 50x4 - 100. The protein solution was then passed repeatedly through the Dintzis column as reported earlier (Ahmad and Salahuddin, 1974). The concentration of isoionic protein solution was determined by the method of Lowry et al., (1951) to the first approximation and its pH was immediately measured on EC digital pH meter.

2. pH measurement

pH of the solution was measured either on EC digital pH meter (serial 022 pH 5651) or on Elico digital pH meter (model Ll - 120). Before measurement, the pH meter was calibrated either with 0.05 M potassium hydrogen phthalate buffer,

pH 4.0, in an acidic pH range or with 0.01 M sodium tetraborate buffer, pH 9.2, in the basic pH range.

3. Optical measurement

Absorbance of the solution in the visible range was measured on a Photochem Colorimeter, model C - 110. In the UV region, light absorption measurements were performed on Cecil UV - Spectrophotometer, model CE 202 and on Cecil UV and Double Beam Spectrophotometer, model CE 594. Fluorescence measurements were carried out on Shimadzu Spectrofluorometer, model RF - 540, in conjunction with fitted Shimadzu Data Recorder, DR - 3, using quartz cell of 1 cm path length.

4. Determination of protein concentration

Protein concentration was determined routinely by the method of Lowry et al., (1951) using crystalline bovine serum albumin as standard and occasionally at 280 nm using the specific extinction coefficient of 6.23 cm^2 g^{-1} (as determined for chymotrypsin inhibitor) and 20.5 cm^2 g^{-1} for chymotrypsin (Wilcox, 1970).

5. Chromatography

a) Ion exchange chromatography

Ion exchange chromatography was carried out on

DEAE - cellulose column (2.4 x 6.0 cm). In order to regenerate DEAE resin, it was suspended in 0.1 N NaOH and washed repeatedly with distilled water till the pH was close to neutral. The resin was then treated with 0.1 N HCl and again washed repeatedly with distilled water. After packing the column, three times bed volume of 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM sodium chloride and 0.02% sodium azide was passed to equilibrate it. The protein was applied on the column with a flow rate of 10 ml/hr and the bound protein was eluted using discontinuous gradient of sodium chloride at a flow rate of 40 ml/hr.

b) Gel chromatography

Hydrodynamic properties of chymotrypsin inhibitor were studied by gel filtration on a Sephadex G - 200 column (2.4 x 78 cm). The column was packed by the method of Ansari and Salahuddin (1973). About 10 grams of gel was allowed to swell in distilled water for about 6 hours in an oven. The fine particles were then removed by decantation. This process was repeated several times until the gel was free from fine particles.

A glass column (2.4 x 90 cm) was taken and washed with detergent, chromic acid and then finally with distilled water. The column was then mounted in a vertical position with help of clamps. Before packing the column, its diameter was determined at different positions along the height of the column. At three places, 2 cm length of graph paper was pasted and column was filled with distilled water. Volume of water corresponding to 2 cm length was collected in three preweighed weighing bottles. The volume of water corresponding to 2 cm length was then determined by dividing the weight of water (W) by its density (d) at room temperature ——

$$V = \pi r^2 l = W/d \qquad (4)$$

$$\text{or } r = \sqrt{\frac{W}{d \pi l}} \qquad (5)$$

where r is the radius of the column. From three independent measurements, the weight of the water was found to be 26.9 grams at temperature 17°C at which density of water is 0.99766 (Merck Index, 1968). The radius of the column computed from equation (5) turns out to be 1.2 cm. Corresponding to bed height of the column as 78 cm, the total

volume of the column was calculated as 353 ml.

After determination of radius of the column a small amount of glass wool, previously boiled in water, was placed at the bottom of the column with the help of a glass rod. Few glass beads were placed on the surface of glass wool. Half of the glass column was then filled with the operating buffer(10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02% sodium azide) keeping the outlet closed. An extension glass column (1.6 x 68 cm) was fitted on the top of the column and the gel slurry was then poured slowly into the column through the extension with the help of a glass rod. As the gel settled down the flow rate was increased gradually from 5 ml/hr to 30 ml/hr. The column was then equilibrated with operating buffer by passing the volume of the buffer equal to three times the bed volume of the column. The packing of the column was checked by passing 0.5% solution of Blue Dextran; the elution pattern shows uniform packing.

The operating buffer on the top of the column was first drained off and 2 ml of the sample

containing 10 mg of protein was applied on the column with the help of an applicator. The stop cock of the column was then opened slowly. As soon as the solution percolated down and no upper liquid layer was visible on the surface, 2 ml of buffer was added from the side of the column and the flow rate was adjusted as 20 ml/hr. The column was then connected to a reservoir containing operating buffer. The fractions of appropriate size (2-5 ml) were collected and monitored by the method of Lowry et al., (1951).

6. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of chymotrypsin inhibitor was performed in Tris glycine buffer, pH 8.2, (I = 0.02) according to the method of Davis (1964). The gel tubes were washed with detergent, chromic acid and then with distilled water. After drying, the tubes were siliconized with 5% dichlorodimethyl silane in chloroform. Two ml of small pore gel solution containing 7% acrylamide, 0.18% N,N' - methylene-bis-acrylamide, 0.03% N,N,N',N' - tetramethylethylenediamine (TEMED) and 0.07% ammonium persulphate was poured

into each tube . After 30 minutes of polymerization 0.5 ml of large pore gel solution was added to each tube. The later contained 2.5% acrylamide, 0.62% N,N' - methylene-bis-acrylamide, 0.06% TEMED, 0.001% riboflavin and 20% sucrose. The gels were kept for 30 minutes for photopolymerization. The protein sample was prepared by adding two drops of glycerol in 1 ml protein solution having a concentration of 1 mg/ml. About 100 µg of protein was applied and electrophoresed for 3 hrs with a current of 2 - 5 mA per tube. The gels were stained with a solutiion of 1% amidoschwarz in 7% acetic acid and destained mechanically with 7% acetic acid.

7. Chemical analysis

(a) Estimation of sulfhydryl groups

The sulfhydryl content of the chymotrypsin inhibitor was determined by the method of Neuman et al.,(1964) using pCMB in 10 mM sodium phosphate buffer, pH 7.0. Chymotrypsin inhibitor (9.4 nM) was incubated with increasing concentration of p-hydroxymercuribenzoate (1.88-28.2 nM) and

increase in absorbance at 250 nm was determined.

b) Determination of carbohydrate content of chymotrypsin inhibitor

i) Neutral hexose

The hexose content of chymotrypsin inhibitor was estimated by the method of Dubois et al., (1956) using galactose as standard.

First 1 ml of 2% (w/v) phenol solution was added to 1 ml carbohydrate or glycoprotein solution followed by the addition of 5 ml concentrated sulphuric acid. After 15 minutes the colour intensity was measured at 490 nm against an appropriate blank of bovine serum albumin. A calibration curve was obtained with galactose (see Fig. 7) and the curve fits the following equation:-

$$(O.D.)_{490} = 5.73 \text{ (mg, galactose)} + 0.1 \qquad (6)$$

ii) Sialic acid

The sialic acid content of chymotrypsin inhibitor was estimated by the method of Warren (1959) using N - acetylneuraminic acid as standard. The standard curve is shown in Fig.8 and the straight line obtained was found to fit the

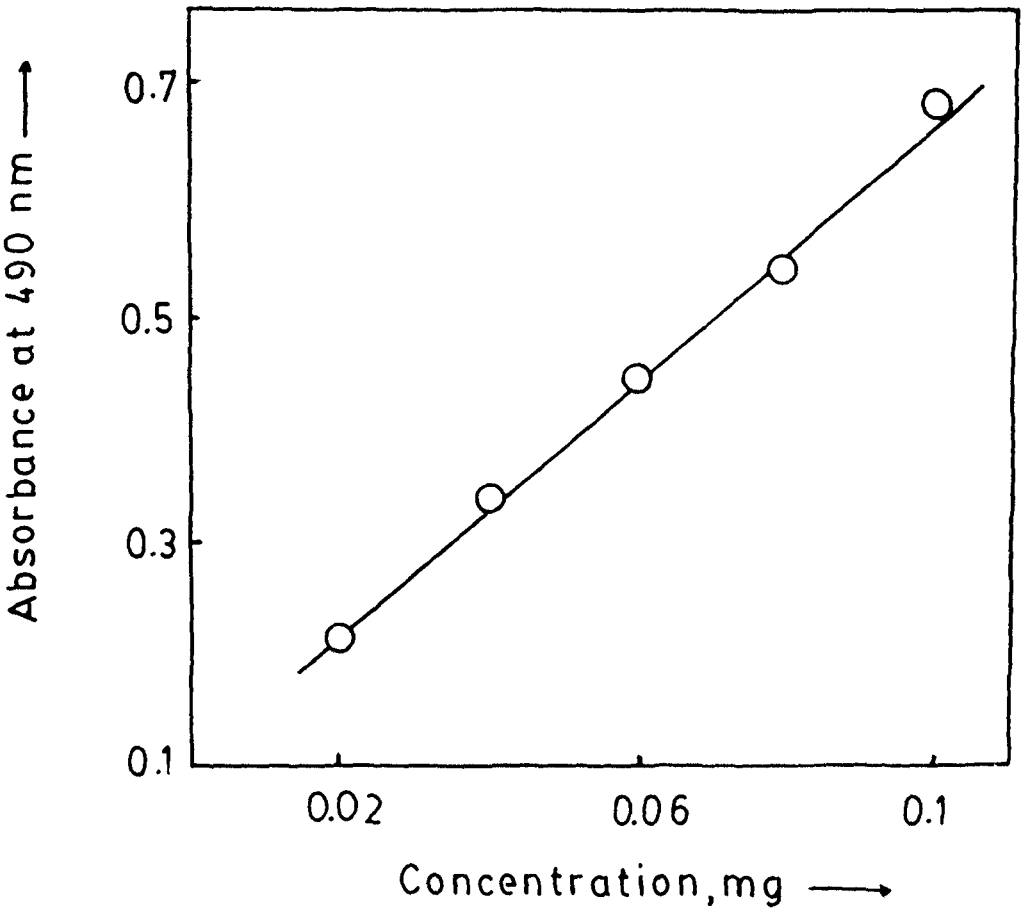


Fig. 7. Calibration curve for the estimation of neutral hexose by the method of Dubois et al., (1956)

Galactose was used as standard. The straight line drawn by the method of least square follows the equation:—

$(O.D.)_{490} = 5.73$ (milligram, galactose) + 0.1

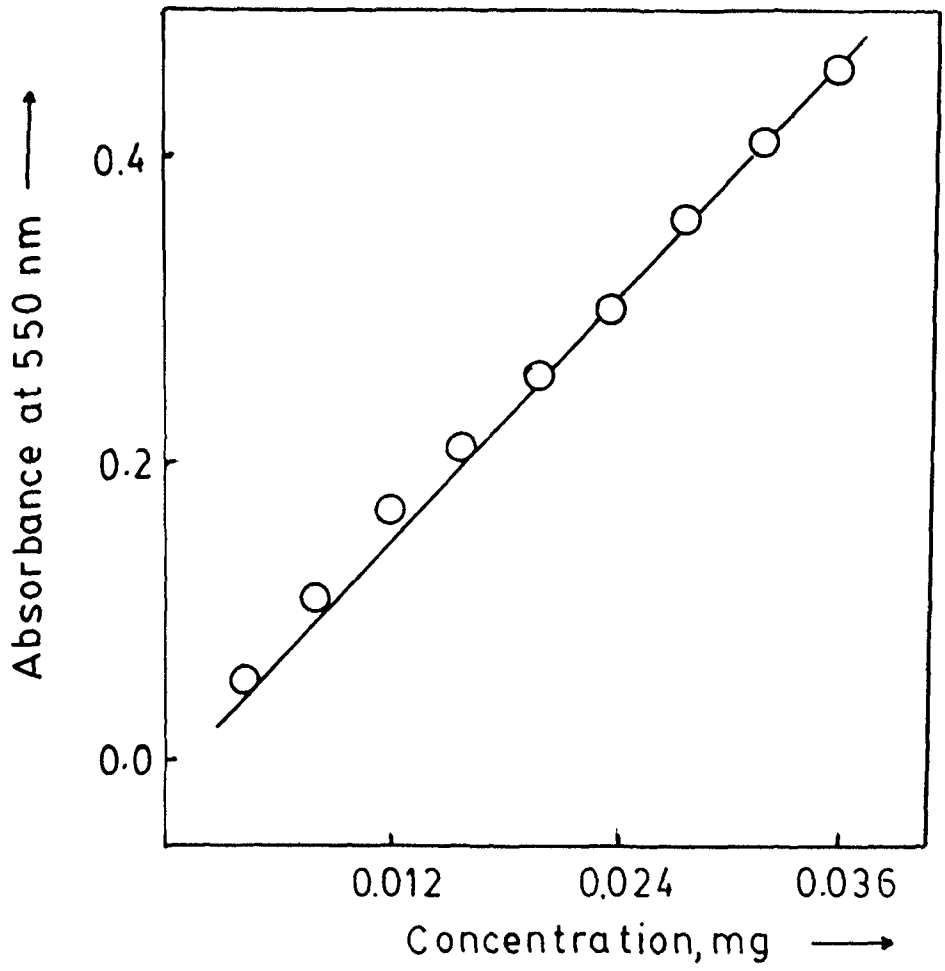


Fig.8. Calibration curve for the estimation of sialic acid by the method of Warren (1959).

N-acetylneuraminic acid was used as standard. The straight line obtained by the method of least square fits the equation:—

$$(O.D.)_{550} = 12.54 \text{ (milligram, sialic acid)} + 8.1 \times 10^{-3}$$

following equation ——

$$(O.D.)_{550} = 12.54(mg,sialic\ acid) + 8.1x10^{-3} \qquad (7)$$

The inhibitor (0.62 mg in 2 ml) was heated with 0.1 N sulphuric acid in a boiling water bath for one hour and the released sialic acid was estimated. For the determination of sialic acid, 0.5 ml of this solution was mixed with 0.1 ml of 0.2 M sodium metaperiodate in 9 M orthophosphoric acid and kept at room temperature for 20 minutes. Then 1 ml of 10% (w/v) sodium arsenite (in a solution of 0.5 M sodium sulphate and 0.1 N H_2SO_4) was added with continuous shaking until a yellow colour appeared and then disappeared. After this 3 ml of 0.6% (w/v) thiobarbituric acid (in a solution of 0.5 M sodium sulphate) was added and the mixture was heated for 15 minutes in a boiling water bath. After cooling equal volume of cyclohexanone was added and centrifuged at 2000 rpm for 20 minutes. The colour intensity of cyclohexanone layer was read at 550 nm using an appropriate blank.

8. Determination of extinction coefficient

Isoionic chymotrypsin inhibitor solution was taken in preweighed weighing bottles and heated to constant weight at 100°C in an oven. After measuring the protein concentration by dry weight method, absorbance of isoionic protein solution was measured at different wave lengths; maximum light absorption occurred at 278 nm. The optical densities of a series of isoionic inhibitor solutions were determined at 278 nm and 280 nm. A linear curve was obtained by plotting absorbance at 278 nm (and at 280 nm) and protein concentration (g/100 ml). The slopes of the curves were computed to be 6.23 and 5.92 at 278 and 280 nm respectively.

9. Determination of molecular weight by sodium dodecyl sulphate polyacrylamide slab gel electrophoresis

The molecular weight of chymotrypsin inhibitor was determined by SDS polyacrylamide slab gel electrophoresis in 12% polyacrylamide gel. Electrophoresis was carried out according to the method of Laemmli (1970) in Tris-glycine buffer (25 mM Tris and 194mM glycine), pH 8.3, containing 0.1% SDS.

The glass plates (192 x 162 x 1.5 mm) were washed first with detergent and then with water. After drying, the plates were siliconized with 5% dichlorodimethyl silane in chloroform. The glass plates were fixed vertically with the help of clamps and sealed with 2% agar solution. About 35 ml of small pore gel solution containing 12% (w/v) acrylamide, 0.6% (w/v) N,N' - methylene-bis-acrylamide, 0.05% (w/v) TEMED, 0.04% ammonium persulphate and 0.14% SDS was poured between the plates. After polymerization the small pore gel was overlaid with a solution of 6% (w/v) acrylamide, 0.13% (w/v) N,N' - methylene-bis-acrylamide, 0.05% TEMED, 0.12% ammonium persulphate and 0.1% SDS. A comb was inserted in the gel solution to form wells. After 20 minutes the comb was removed and the wells were layered with distilled water.

The sample was prepared by heating protein in sample buffer (0.06 M Tris - HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS and 0.05% (w/v) bromophenol blue). The protein solutions were heated in a boiling water bath for 20 minutes. After cooling, 0.02 M 2-mercaptoethanol was added, if needed. The protein sample

(0.01 - 0.5 ml) containing 25 - 60 µg of protein was applied in the well and gently layered with electrophoretic buffer. The electrophoresis was performed with a current of 2-4 mA per lane for about three hours.

Staining procedure

The gels were stained for protein and glycoprotein with coomassie brilliant blue R and Schiff reagent respectively.

i) Staining procedure for protein

For staining of protein, the dye solution was prepared by dissolving 1% coomassie brilliant blue R - 250 in a mixture of 50% (v/v) methanol, 10% acetic acid and water (Weber and Osborn, 1969). The gel was dipped in staining dye for 1 hr and destained mechanically with 10% acetic acid.

ii) Staining procedure for glycoprotein

Gels were stained for carbohydrate using PAS - staining procedure. SDS was removed before staining by carrying out following steps ———

a) 25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid ; overnight

b) 10% (v/v) isopropyl alcohol and 10% (v/v) acetic acid; 6 - 9 hrs.

c) 10% (v/v) acetic acid; overnight.

The fixed gels were treated as follows using at least 100 ml of rinsing solution at each stage ——

a) 0.5% (w/v) periodic acid; 2 hrs

b) 0.5% (w/v) sodium arsenite and 50% (v/v) acetic acid; 1 hr

c) 0.1% (w/v) sodium arsenite and 5% (v/v) acetic acid; 20 minutes

d) 10% (v/v) acetic acid; 20 minutes

e) Schiff reagent; overnight

f) 0.1% (w/v) sodium metabisulphite in 0.1 N HCl

The last step was repeated three or four times until a pink colour band was appeared.

10. Measurement of inhibitory activity

The activity of chymotrypsin inhibitor was measured against chymotrypsin and trypsin using casein as substrate.

The proteinase activity was determined by the method of Anson (1938) with TLCK treated chymotrypsin or trypsin. A standard assay mixture contained enzyme (0.01%, w/v) and 1% (w/v) casein in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. The mixture was incubated

at 37°C for 1 hr. The caseinolytic activity was measured after stopping reaction with 10% TCA. In control 10% TCA was added to the enzyme solution before the addition of casein. The proteinase activity was measured both in presence and absence of chymotrypsin inhibitor.

11. Heat treatment of chymotrypsin inhibitor

Two ml of chymotrypsin inhibitor solutions (1.5 mg/ml) in 10 mM sodium phosphate buffer, pH 7.5, containing 150 m M sodium chloride and 0.02% sodium azide were heated in a water bath at 45°C, 50°C, 55°C and 60°C. After incubation for 15 minutes, the solutions were immediately cooled in ice cold water and stored at 4°C. The inhibitory activity of different preparations at different concentrations were measured using casein as substrate.

12. Isolation and purification of goat chymotrypsin inhibitor

Goat plasma was collected and its protein content was determined. Solid ammonium sulphate was added to obtain 50% saturation and the pH of the solution was adjusted to 4.5 with 4N H_2SO_4. The precipitated prortein was removed by centrifugation

and salt concentration of supernatant was raised to 80%. The precipitated protein was extensively dialyzed and then fractionated by ion exchange chromatography on DEAE - cellulose column equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM sodium chloride and 0.02% sodium azide. The bound protein was eluted using discontinuous gradient which is achieved by adding different concentrations of sodium chloride (50 mM - 150 mM) to 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM sodium chloride and 0.02% sodium azide. The protein fractions showing antichymotryptic activity were pooled and rechromatographed on the same column.

RESULTS

1. Isolation of chymotrypsin inhibitor:

Goat chymotrypsin inhibitor was isolated both from serum and plasma by salt fractionation and was further purified by ion exchange chromatography on DEAE-cellulose column.

Protein concentration of goat plasma was estimated by the method of Lowry et al., (1951) on seven samples obtained at different time spreading over a period of two years and the results showed the presence of 5.3 - 6.7% protein in the goat plasma; the corresponding value in serum was found to be about 5%. Different steps in isolation and purification of chymotrypsin inhibitor from goat plasma are summarized in Table VIII. Most of the plasma proteins including albumin were precipitated with 50% ammonium sulphate at pH 4.5 and the supernatant containing 1.1 g protein was made 80% with respect to ammonium sulphate. The precipitated protein (763 mg) as well as the protein in supernatant (176 mg) were checked for antichymotryptic activity. The inhibitory activity against chymotrypsin was measured as described in experimental section and it was located only in the

TABLE VIII

ISOLATION AND PURIFICATION OF GOAT CHYMOTRYPSIN INHIBITOR

Isolation Steps	Total Protein	% protein yield	% inhibitory activity per mg of protein
1. Plasma	53 g	100	5.0
2. 80% ammonium sulphate saturation --			
i) precipitate	763 mg	1.4	16
ii)supernatant	176 mg	0.33	--
3. Ion exchange chromatography on DEAE - cellulose column (peak III)	80 mg	0.15	100

precipitate. The precipitated protein was then fractionated by ion exchange chromatography on DEAE - cellulose column (2.4 x 6.0 cm) in 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM sodium chloride and 0.02% sodium azide. The bound protein was eluted batchwise with the buffer containing different concentrations of sodium chloride as shown in Fig. 9. The protein eluted with the buffer containing 50 mM sodium chloride was devoid of antichymotryptic activity and found 39% of the total protein applied on the column. About 23 mg protein was eluted with buffer containing 100 mM sodium chloride; the protein fractions did not show any antichymotryptic activity. Likewise peak III and a small peak IV were obtained with 150 mM and 200 mM sodium chloride, the activity was located in peak III which contained 11.5% of the total protein applied on the column. The peak III was rechromatographed on the same column and the elution profile is shown in Fig.10. Thús about 80 mg of chymotrypsin inhibitor was obtained from one litre of goat plasma. Our results are comparable to those obtained by Abdullah et al., (1983) who isolated 80 mg of $alpha_1$-anti-chymotrypsin from one litre human serum by salt fractionation , ion exchange and affinity chromatography, but

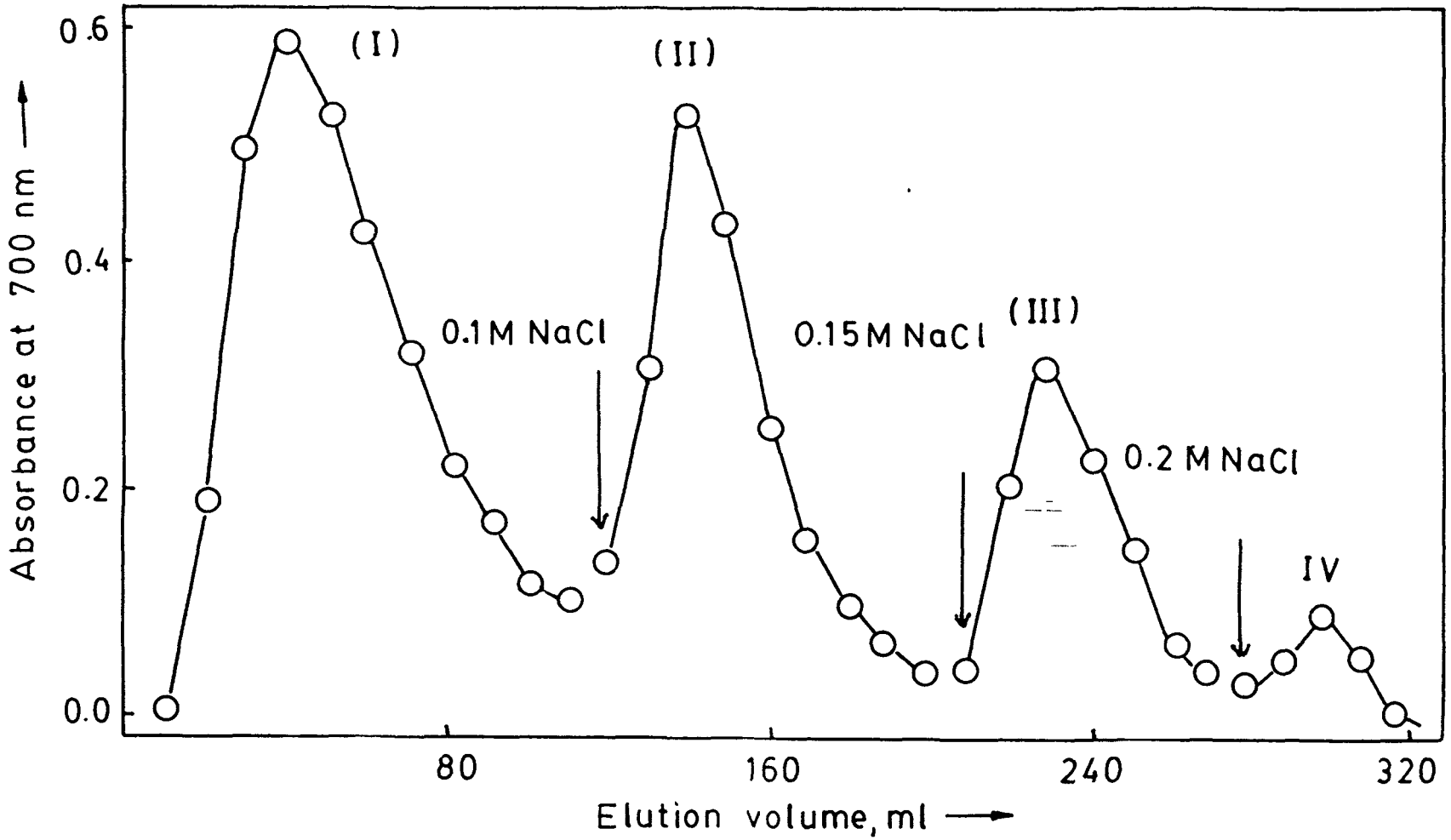


Fig. 9. Chromatographic profile of 80 % ammonium sulphate fraction of goat plasma on DEAE - cellulose column.

About 80 mg of protein was applied on DEAE - cellulose column (2.4 × 6 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5 containing 50 mM sodium chloride and 0.02 % sodium azide. The bound protein was eluted using discontinuous sodium chloride gradient (100 mM - 200 mM) in 10 ml fractions at a flow rate of 40 ml/hr. The column was monitored by the method of Lowry et al., (1951).

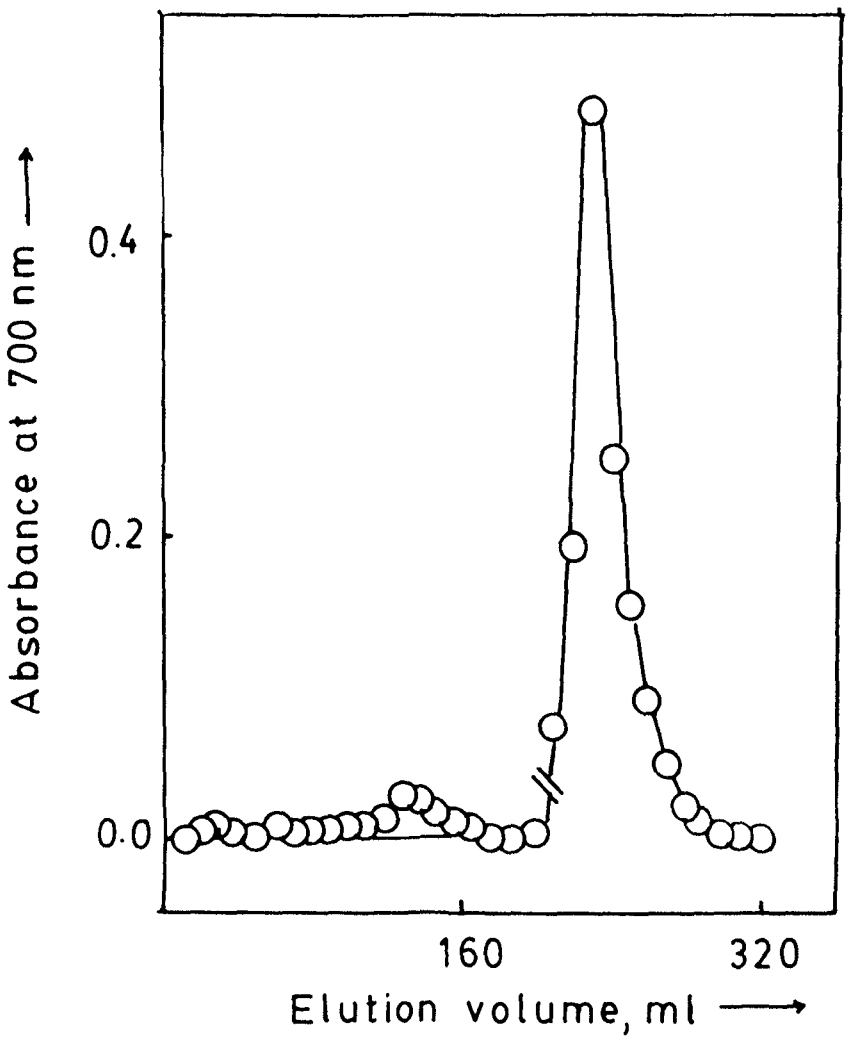


Fig. 10. Rechromatography of 80% ammonium sulphate fraction on DEAE-cellulose column.

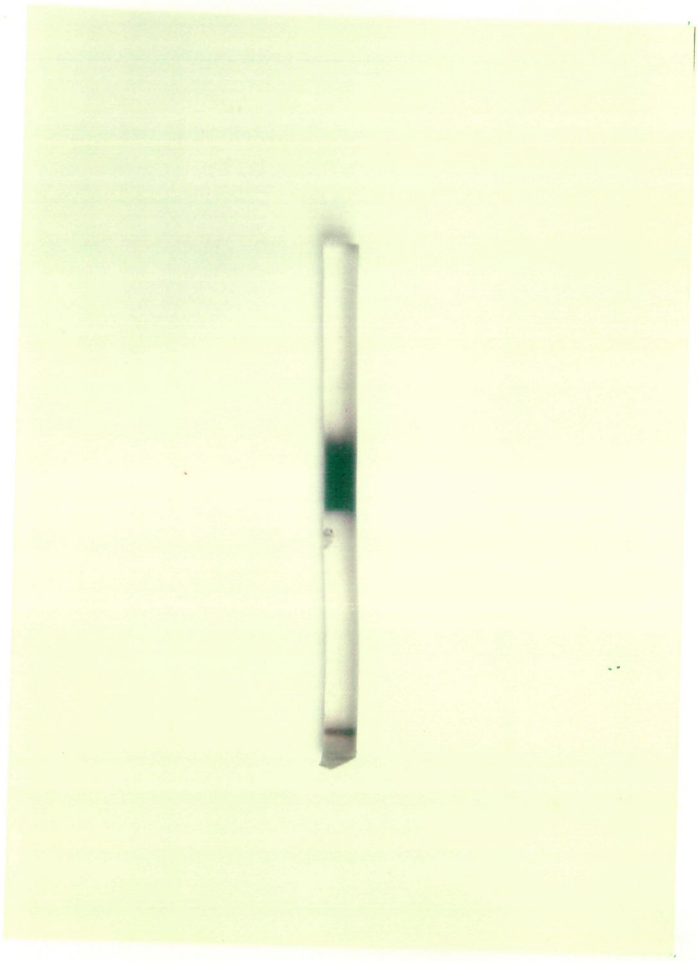
The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM sodium chloride and 0.02 % sodium azide. About 30 mg of protein collected from the fractions under peak III of Fig. 1 was applied on the column and the bound protein was eluted with 10 mM sodium phosphate buffer containing 0.1 M & 0.15 M NaCl at a flow rate of 40 ml/hr. Each fraction contains 10 ml eluant. The column was monitored by the method of Lowry et al., (1951).

substantially lower than that (250-300 mg) found by Laine et al., (1984b). However, the yield obtained in this study was substantially higher than that (35-40 mg)found by Siddiqui et al., (1980) and Katsunuma et al., (1980).

When purified chymotrypsin inhibitor was electrophoresed in Tris glycine buffer (4.95 mM Tris and 38.4 mM glycine), pH 8.2, in 7% polyacrylamide gel, a single band with R_m value of 0.48 was obtained (see Fig.11) The zone broadening may be attributed due to glycoprotein nature of inhibitor as it was found to have about 17% carbohydrate including 5% sialic acid. When the inhibitor was passed on a Sephadex G-200 column equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02% sodium azide, it eluted as a single symmetrical peak (see Fig. 12). This observation taken together with the fact that the inhibitor moved as a single band in sodium dodecyl sulphate polyacrylmide gel electrophoresis (see Fig. 13) showed that our inhibitor preparation is homogeneous with respect to size.

Fig. 11. Polyacrylamide gel electrophoretic pattern of chymotrypsin inhibitor.

About 100 µg of protein was electrophoresed in 7% gel for 3 hrs in Tris glycine buffer, pH 8.2, (I = 0.02) using a current of 2 - 5 mA per tube. The gels were stained with amidoschwarz and destained mechanically with 7% acetic acid.



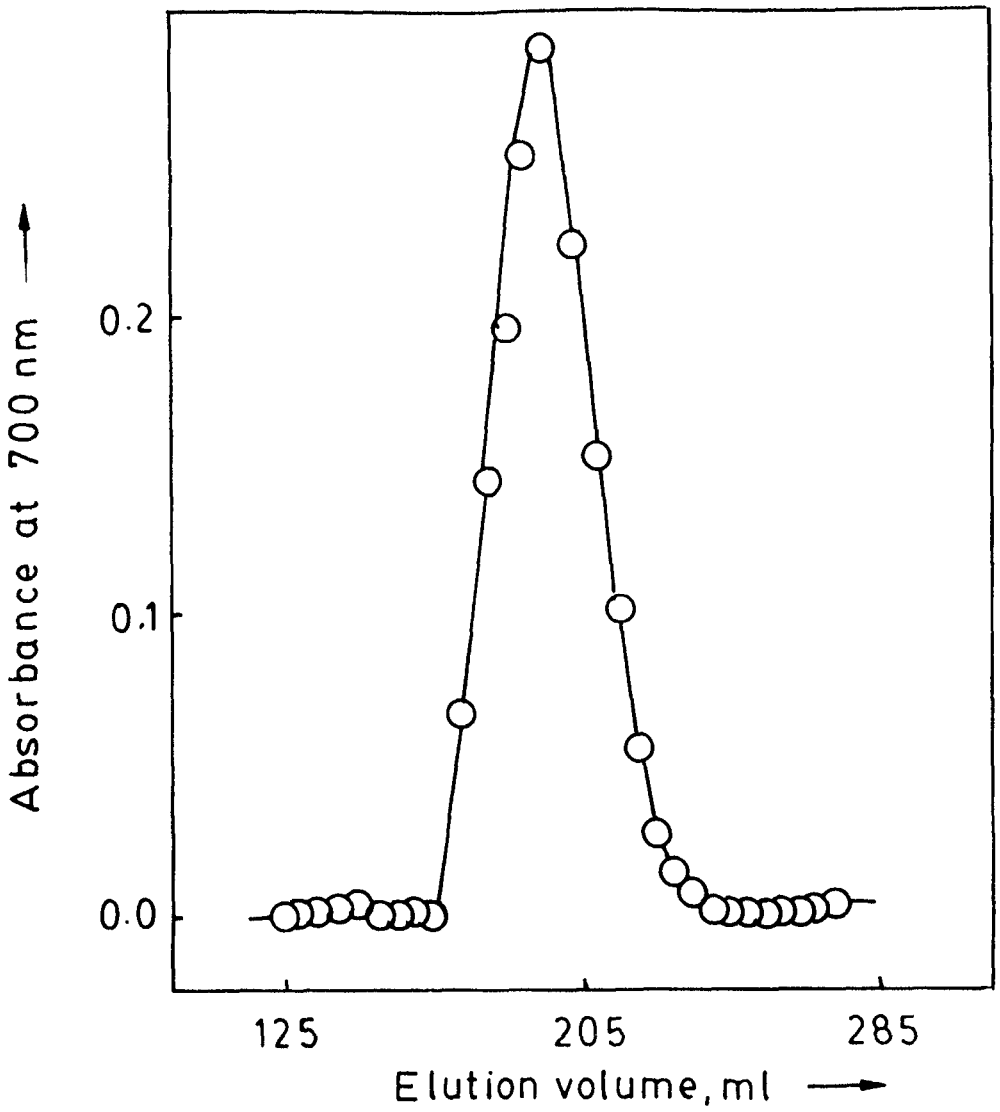
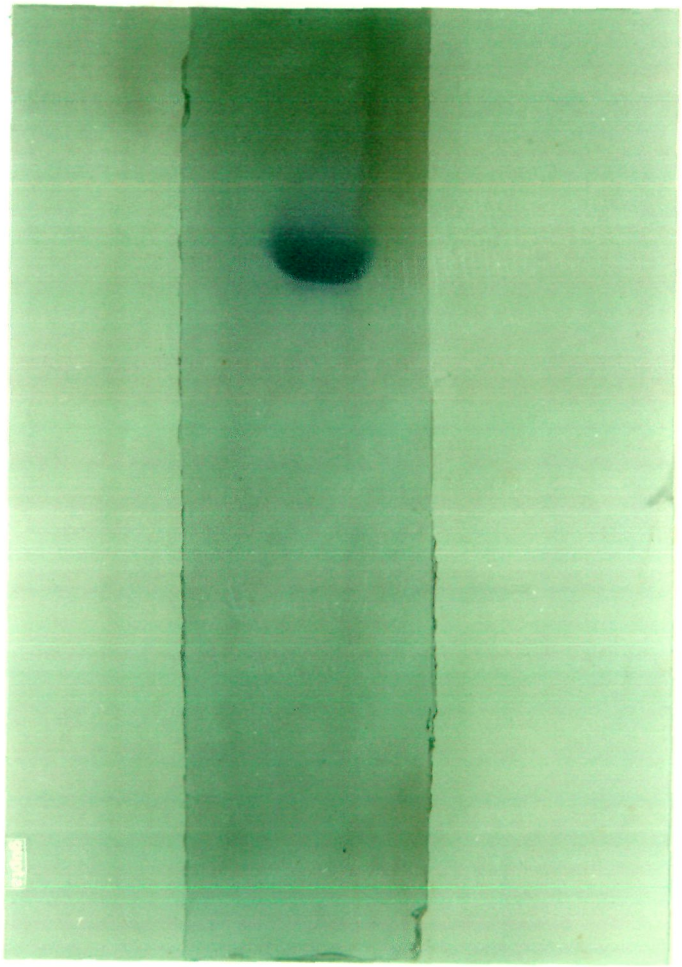


Fig.12. Gel chromatography of chymotrypsin inhibitor.

About 5mg of purified chymotrypsin inhibitor obtained by ion exchange chromatography (peak A of Fig. 2) in 3ml 10mM sodium phosphate buffer, pH 7.5, containing 150mM NaCl and 0.02% spdium azide was applied on Sephadex G-200 column (2.4 ×78 cm) which was previously equilibrated with the same buffer. The protein was eluted in 5ml fractions with operating buffer at a flow rate of 20ml/hr. The column was monitored by the method of Lowry et al., (1951).

Fig. 13. Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis of chymotrypsin inhibitor.

About 50 µg of the purified chymotrypsin inhibitor was electrophoresed on 12% polyacrylamide slab gel in the presence of Tris glycine buffer (25 mM Tris and 194 mM glycine), pH 8.3, containing 0.1% SDS for about 3 hrs using a current of 2 - 4 mA per lane. The gel was stained with coomassie brilliant blue and destained mechanically with 10% acetic acid.



2. **Determination of molecular weight:**

The molecular weight of chymotrypsin inhibitor was determined by sodium dodecyl sulphate polyacrylamide gel electrophresis in Tris-glycine buffer (25 mM Tris and 194 mM glycine), pH 8.3, containing 0.1% SDS according to the method of Laemmli (1970).

The marker proteins used including transferrin, bovine serum albumin, ovalbumin, Ig G (heavy and light chain), chymotrypsinogen A, cytochrome c and inhibitor were electrophoresed in identical conditions. The R_m of marker proteins and inhibitor were determined as the movement of protein band relative to the mobility of dye. Here R_m is determined with a maximum error of 6%. The results are summarized in Table IX. A plot of log M versus R_m was drawn by the method of least square (see Fig. 14) and the straight line obtained fits the equation :--

$$\log M = - 1.39 \ R_m + 5.19 \qquad (8)$$

Corresponding to R_m of 0.26 for chymotrypsin inhibitor, the molecular weight of inhibitor was calculated with the help of above equation as 68 kDa. The maximum uncertainty in the molecular weight determination was 8%. The value of molecular weight for chymotrypsin inhibitor which we have obtained is in agreement with that reported

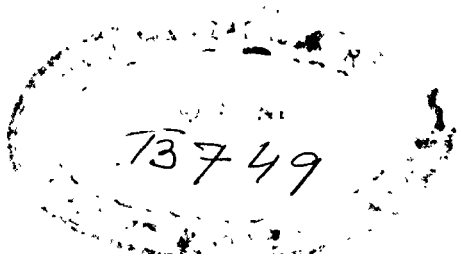


TABLE IX

LOG M AND R_m VALUES FOR MARKER PROTEINS AND INHIBITOR USED IN SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

S.N.	Proteins	Molecular Weight (kDa)	Log M	R_m
1.	Transferrin	76.5[a]	4.8837	0.22
2.	Bovine serum albumin	68[a]	4.8325	0.27
3.	Ig G,heavy chain	51[b]	4.7076	0.33
4.	Ovalbumin	43[a]	4.6335	0.39
5.	Chymotrypsinogen A	25.7[a]	4.4099	0.6
6.	Ig G,light chain	25[b]	4.3979	0.57
7.	Cytochrome c	11.7[a]	4.0682	0.77
8.	Chymotrypsin inhibitor	-	-	0.26

References: [a] Hames (1986)

[b] Stanworth and Turner (1986)

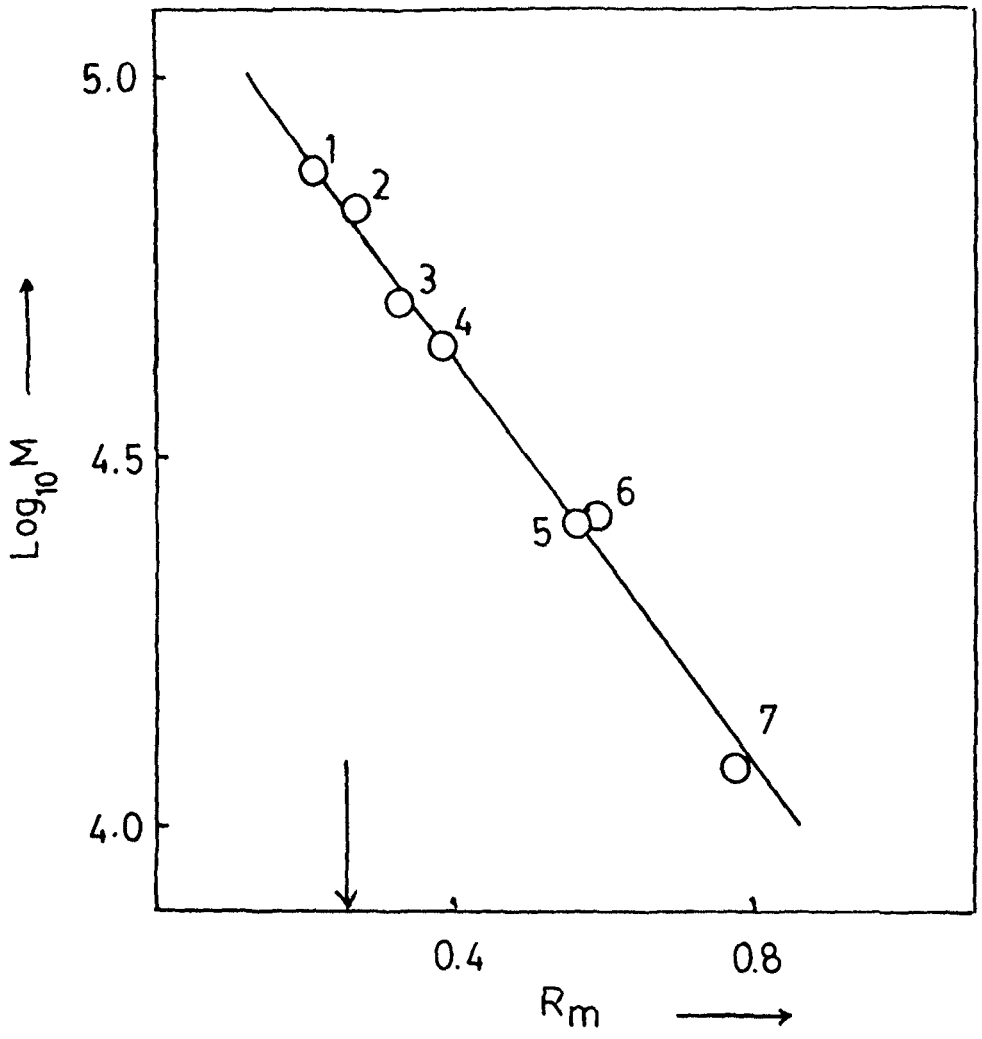


Fig. 14. Plot of R_m values of marker proteins versus logarithm of molecular weight.

The marker proteins were :- (1) transferrin (76.5 kDa), (2) bovine serum albumin (68 kDa), (3) heavy chain of α - globulin (51 kDa), (4) ovalbumin (43 kDa), (5) light chain of γ - globulin (25 kDa), (6) chymotrypsinogen A (25.7 kDa) and (7) cytochrome c (11.7 kDa). The R_m of chymotrypsin inhibitor is indicated by an arrow.

for alpha$_1$-antichymotrypsin by Travis et al., (1978b) but slightly higher to that obtained by Laine et al., (1984b).

3. **Optical properties of chymotrypsin inhibitor:**

The concentration of isoionic preparation of chymotrypsin inhibitor solution was 1.6 mg/ml and the pH of the solution was measured to be 5.5. This cannot be compared with the literature value since isoionic pH of chymotrypsin inhibitor is being reported for the first time in this study.

The UV absorption spectra of chymotrypsin inhibitor in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride is shown in Fig.15. It can be seen that maximum absorption occurred near 278 nm. Protein solutions of varying concentrations (0.07-0.16 mg/ml) were prepared from isoionic preparation of the inhibitor and their absorbance were measured at 278 and 280 nm. The slope of the curve drawn between absorbance and protein concentration in g/100 ml, as determined by the method of least square analysis, was found to be 6.23 at 278 nm (see Fig. 16) and 5.92 at 280 nm (see Fig. 17). The specific extinction coefficient of human plasma alpha$_1$-antichymotrypsin was determined by Travis et al.,

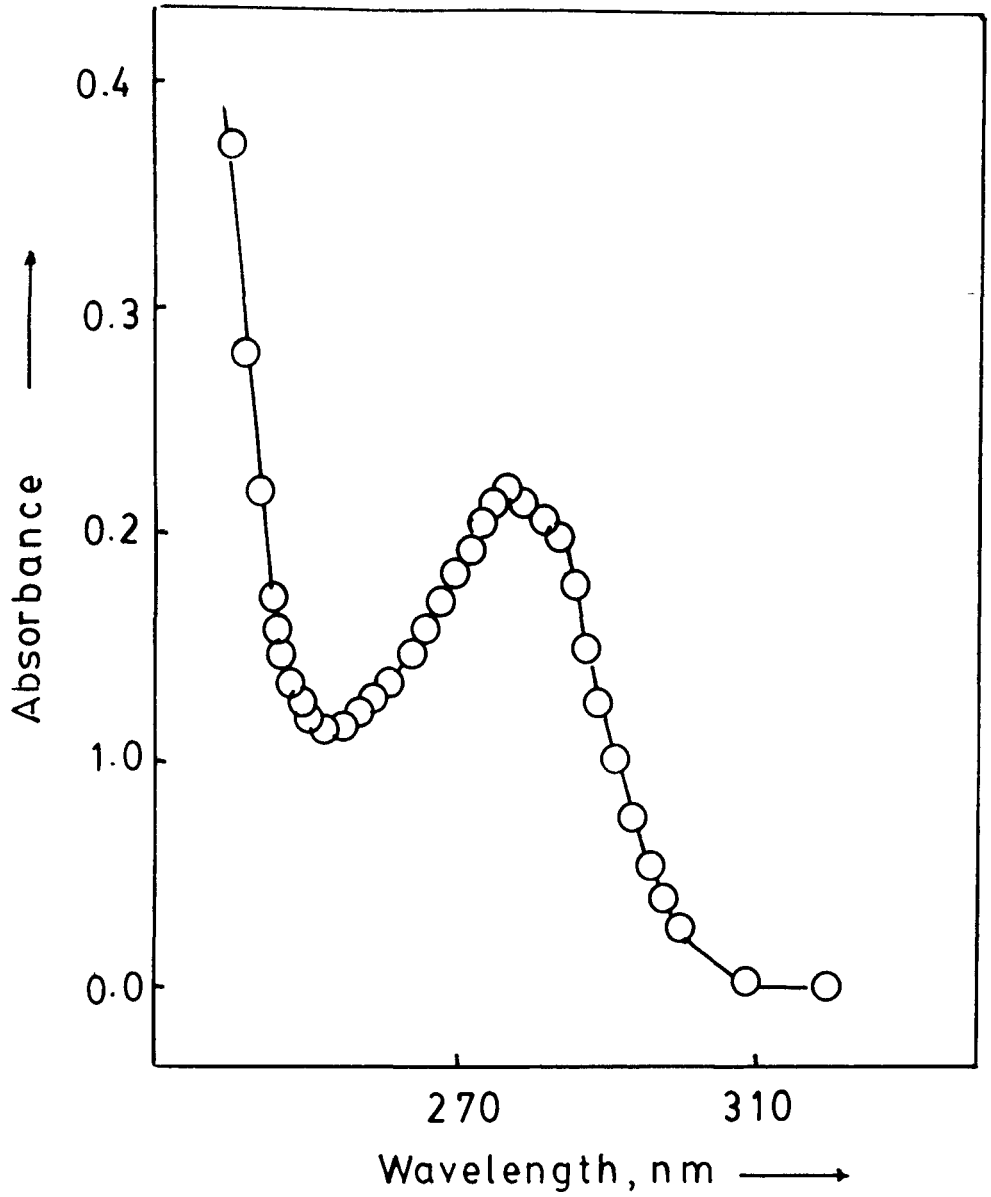


Fig. 15. Ultraviolet absorption spectra of chymotrypsin inhibitor.

The solution contains 0.11 mg / ml of the inhibitor in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02 % sodium azide.

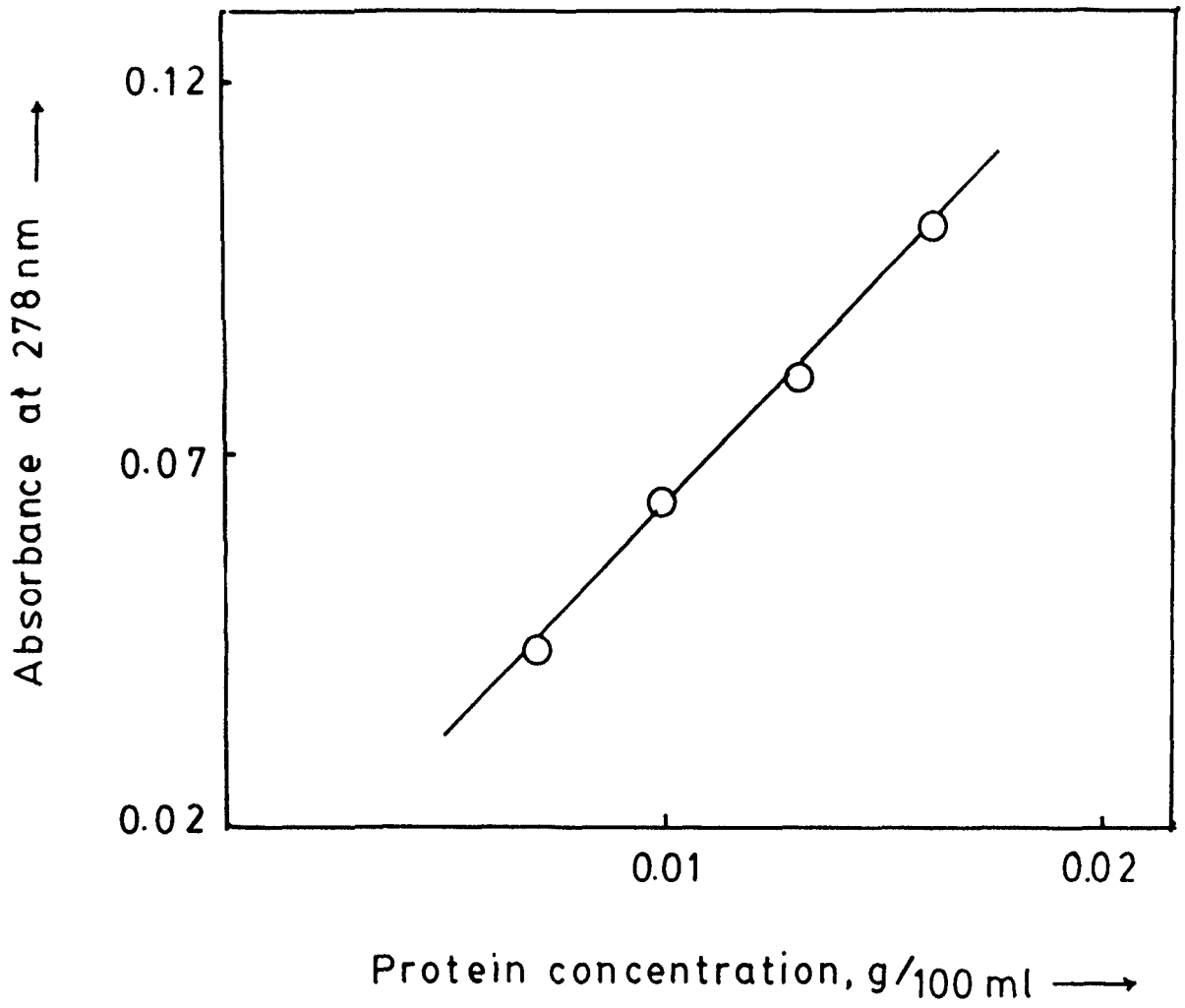


Fig. 16. Determination of specific extinction coefficient of the chymotrypsin inhibitor at 278 nm in deionized water.

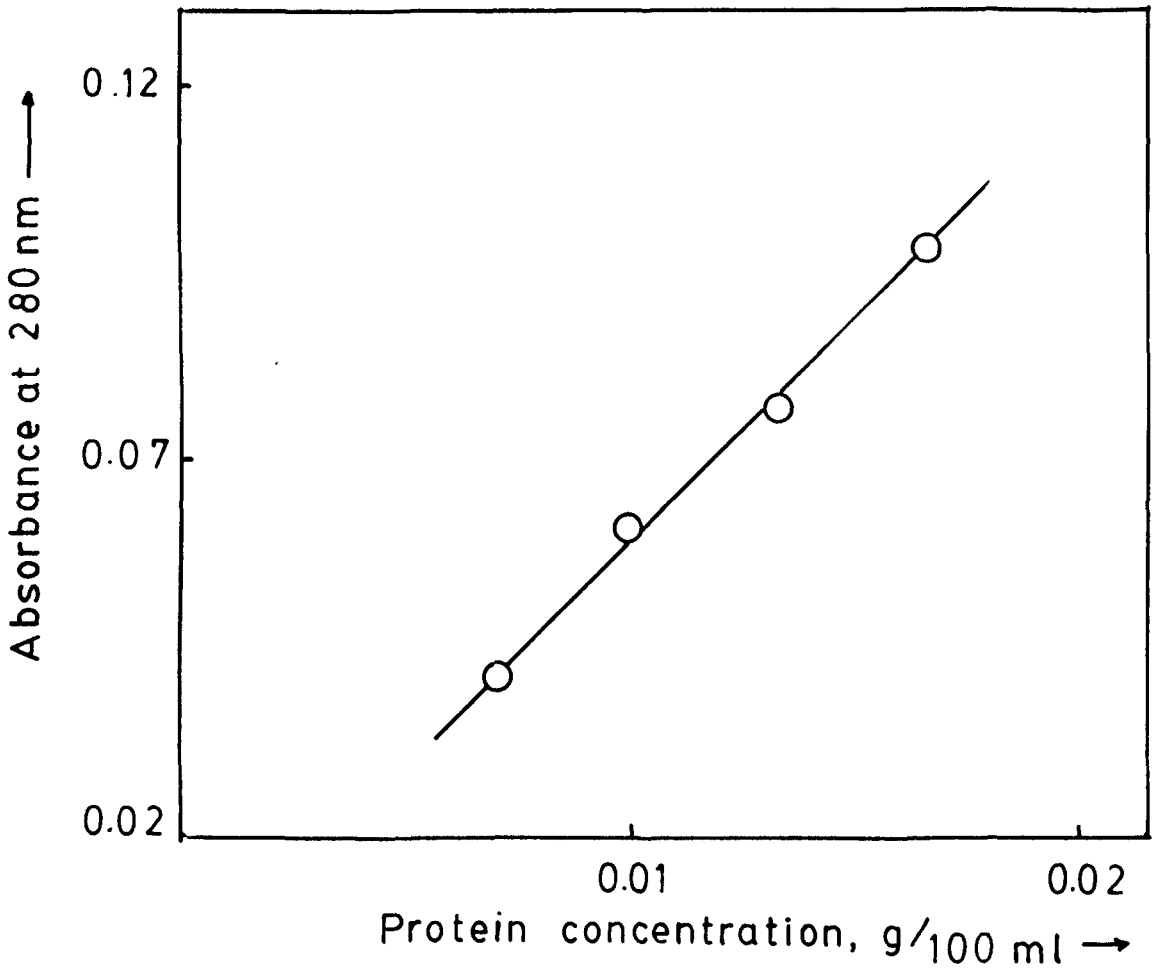


Fig.17. Determination of specitic extinction coefficient of chymotrypsin inhibitor in deionized water.

(1978b) to be 6.2 cm^2 g^{-1} at 280 nm which is only 5% higher than that found in this study.

The excitation and emission spectra of chymotrypsin inhibitor were measured in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride at a protein concentration of 0.02 mg/ml. The results are shown in Fig 18; emission and excitation maxima were found to be 338 and 278 nm respectively. These spectral features suggest that inhibitor is a tryptophan containing protein.

4. **Chemical analysis:**

i) Carbohydrate content

The fact that chymotrypsin inhibitor band in sodium dodecyl sulphate polyacrylamide gel electrophoresis was stainable with periodic acid Schiff reagent indicates glycoprotein nature of inhibitor.

Neutral hexose content of the chymotrypsin inhibitor was determined by the method of Dubois et al., (1956) to be 12% with an experimental error of 10% . This would corresopond to 46 hexose residues per mole of inhibitor. The sialic acid content of the inhibitor was determined by the method of Warren

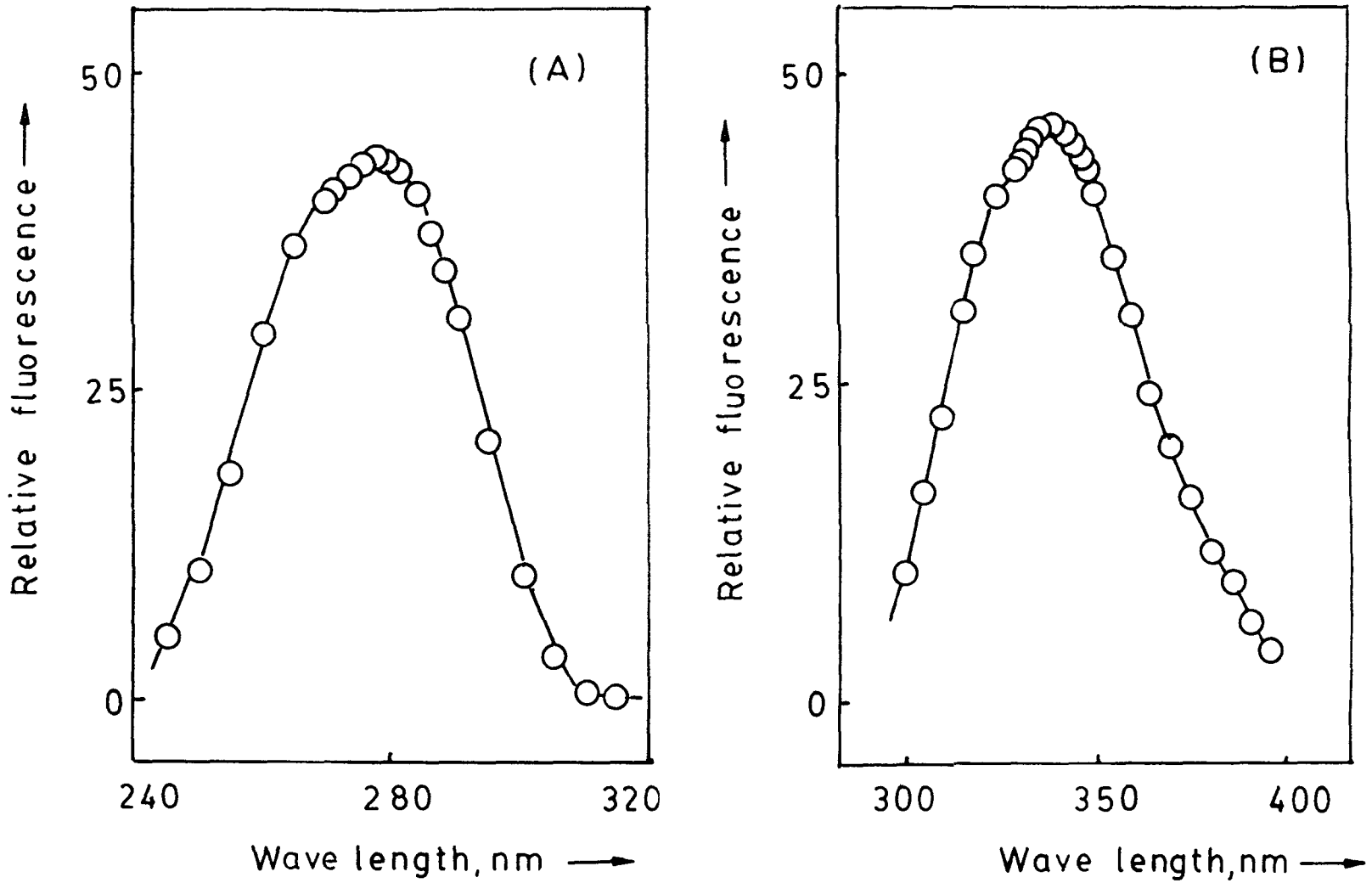


Fig.18. Excitation (A) and emission (B) spectra of chymotrypsin inhibitor.

The inhibitor was taken in the same buffer as in Fig.17 but the concentration was 0.02 mg / ml. The spectra were recorded at room temperature using 10 nm slit

(1959) and the value was found to be 4.5% i.e. 10 sialic acid residues per mole of inhibitor.

The carbohydrate content of human alpha$_1$-antichymotrypsin has been determined by different groups (see Table X). The reported value for neutral hexose content lies between 10-11% i.e. 38-41 residues per mole of inhibitor. The sialic acid content of the chymotrypsin inhibitor has been reported in the range of 4-6% corresponding to 11-14 moles of sialic acid per mole of inhibitor. From the results it seems that human and goat chymotrypsin inhibitors do not differ significantly in neutral hexose and sialic acid content.

ii) Estimation of thiol groups

The thiol groups present in chymotrypsin inhibitor was determined in 10 mM sodium phosphate buffer, pH 7.0 and the results are graphically shown in Fig. 19 which depicts a plot between increase in the absorbance at 250 nm due to reaction of pHMB with thiol groups of inhibitor and molar ratio of pHMB to inhibitor. The inflection point occurred at pHMB to inhibitor molar ratio of one suggesting the presence of a single sulfhydryl group per mole of inhibitor.

TABLE X

CARBOHYDRATE COMPOSITION OF CHYMOTRYPSIN INHIBITOR

Plasma/ Serum	Neutral hexose		Sialic acid		Reference
	Percent	moles/ mole protein	Percent	moles/ mole protein	
Goat	12	47	4.5	10	This study
Human	-	41	-	14	Heimburger et al.,(1971)
	10.9	38-40	5.8	11-14	Travis et al., (1978 b)
	10.3	-	4.51	-	Laine and Hayem (1981)

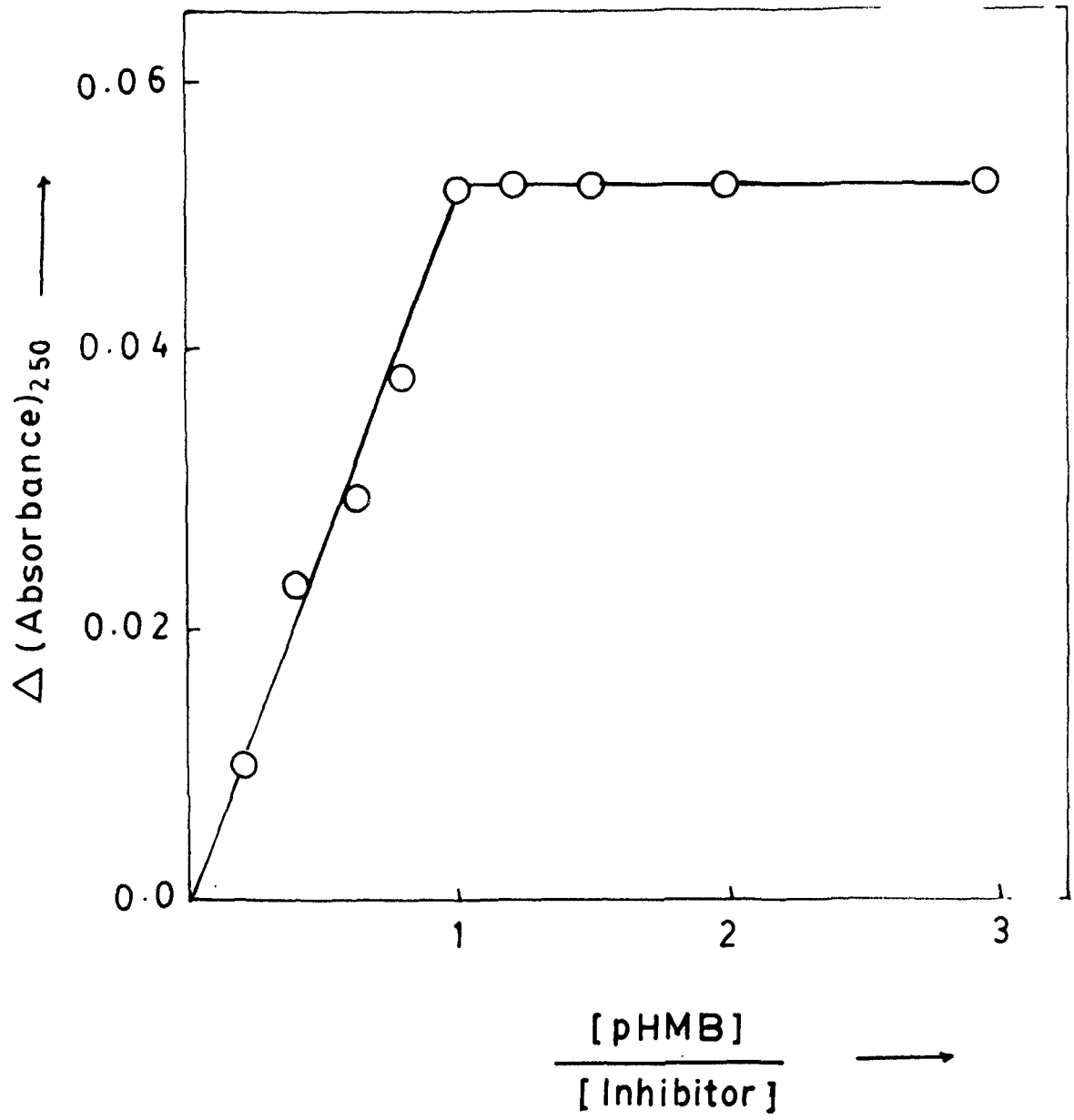


Fig. 19. Titration of thiol groups of chymotrypsin inhibitor.

A fixed concentration of inhibitor (47.2 nM) in 10mM sodium phosphate buffer, pH 7.0, was incubated with increasing concentration of pHMB (9.44 nM – 131.6 nM) for 6 hr; the increase in the absorbance at 250 nm was recorded.

However, after modification of this sulfhydryl group with pHMB, no effect on its inhibitory activity was found.

Conflicting reports are available in the literature regarding the number of cysteine residues in human plasma/serum $alpha_1$-antichymotrypsin. Heimburger et al., (1971) and Laine and Hayem (1981) could not detect any cysteine residue by amino acid analysis while using the same procedure Travis et al., (1978b) found two cysteine residues per mole of inhibitor. From the analysis of cDNA for human $alpha_1$-antichymotrypsin Chandra et al., (1983) deduced two cysteine residues per mole of inhibitor. We can not compare our results with the literature value as we have determined the number of sulfhydryl groups in native condition, therefore, the completely burried thiol groups may remain untitrable.

5. **Hydrodynamic properties:**

Hydrodynamic properties of goat chymotrypsin inhibitor were determined by analytical gel chromatography on a Sephadex G-200 column (2.4 x 78 cm) in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride

and 0.02% sodium azide.

The void volume of the column was determined by passing a band of Blue Dextran and the elution profile is shown in Fig. 20. The elution volume of Blue Dextran which is equal to the void volume of the column was found to be 140 ml. The inner volume, V_i, was determined by passing 0.5% glucose solution. The elution profile is shown in Fig. 21 and its elution volume is given by :--

$$V_{e,g} = V_o + K_d V_i \qquad (9)$$

The value for distribution coefficient, K_d, for glucose is 1 and the value of $V_{e,g}$ was calculated to be 340 ml. With these values of $V_{e,g}$, V_o and K_d, the value of V_i was computed to be 200 ml. The distribution coefficient, K_d and available distribution coefficient, K_{av}, for the marker proteins and inhibitor were calculated using the following expressions:-

$$K_d = (V_e - V_o) / V_i \qquad (10)$$

$$K_{av} = (V_e - V_o) / (V_t - V_o) \qquad (11)$$

The total volume, V_t, of the column was determined to be 353 ml (see experimental section). The

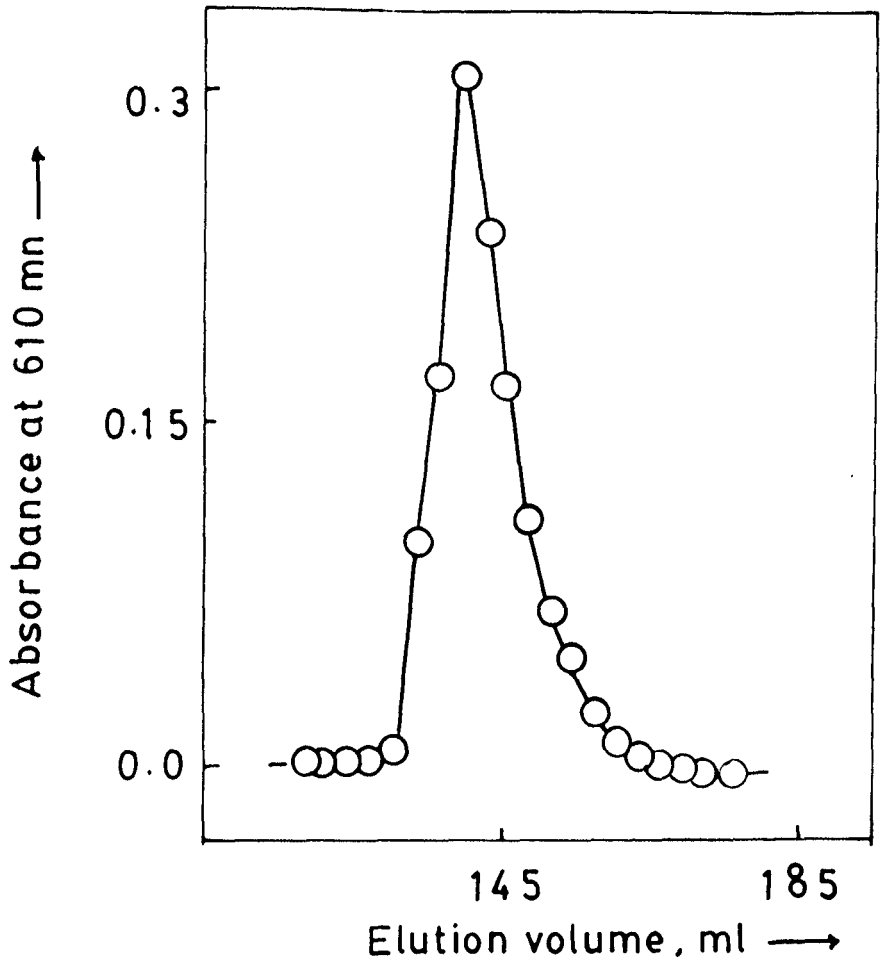


Fig.20. Elution profile of Blue Dextran on Sephadex G-200 column.

About 10 mg of Blue Dextran was applied on the column equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02 % sodium azide and eluted in 3 ml fractions at a flow rate of 20 ml/hr with equilibrating buffer. The colour intensity was read at 610 nm.

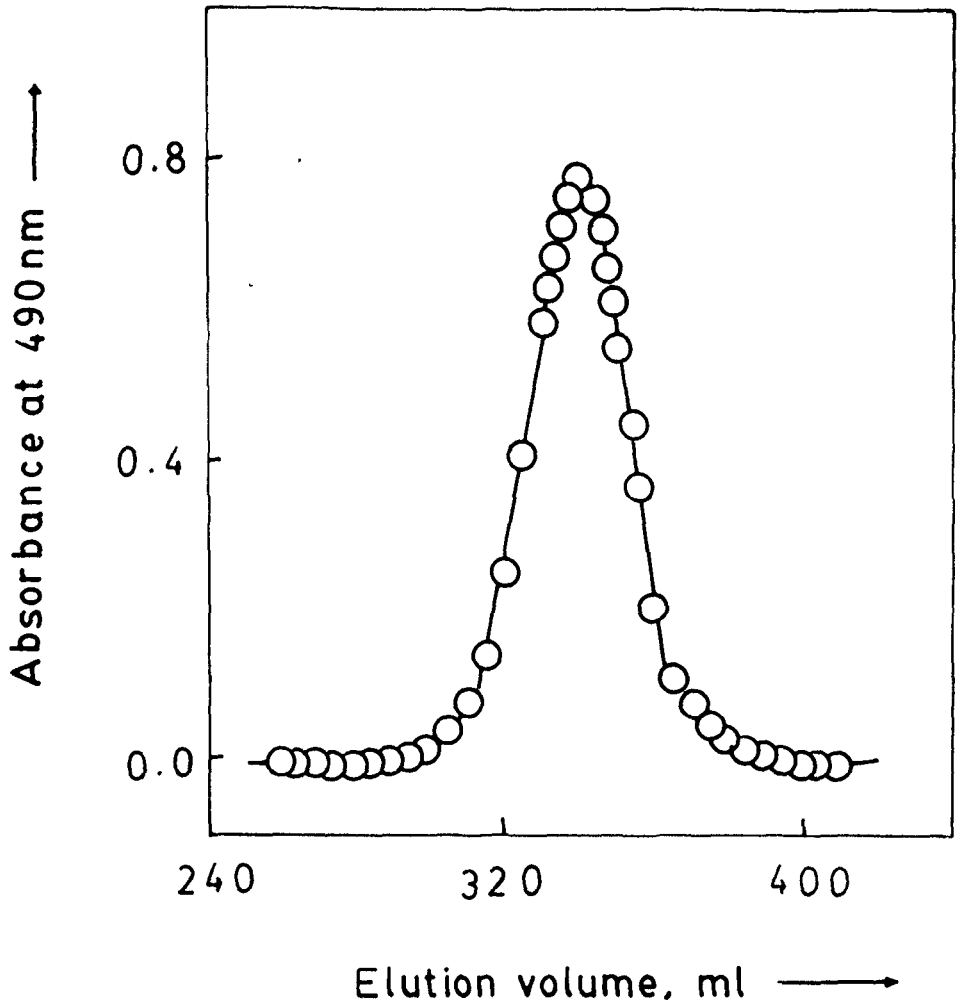


Fig. 21. Elution profile of glucose on Sephadex G-200 column.

About 10 mg of glucose in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02 % sodium azide was applied on the column and the carbohydrate was eluted in 2 - 5 ml fractions at a flow rate of 20 ml / hr. The column was monitored by the method of Dubois et al., (1956).

marker proteins used in gel filtration along with their molecular weights and Stokes radii are listed in Table XI. The elution profiles of different marker proteins and chymotrypsin inhibitor are depicted in Figs. 22-24. The elution volume of goat plasma chymotrypsin inhibitor was calculated as 202 ±1 ml from the average of four readings.

For the determination of molecular weight, the gel filtration data were analyzed by the method of least square using the following expressions:-

$$V_e / V_o = A - B \log M \qquad (12)$$

$$M^{1/3} = A_1 - B_1 K_d^{1/3} \qquad (13)$$

where M is the molecular weight of the inhibitor. The linear plots according to equations (12) and (13) are depicted in Figs. 25 and 26. The values of V_e / V_o, log M, $K_d^{1/3}$ and $M^{1/3}$ for marker proteins and inhibitor are given in Table XII and of constants A, B, A_1 and B_1 are given in the legends of Figs.25 and 26. The value of V_e/V_o of 1.44 for chymotrypsin inhibitor corresponds, according to Fig. 25 to a molecular weight of 78 kDa. From a linear plot shown in Fig. 26 the molecular weight of the inhibitor was found to be 74 kDa. The average molecular weight of chymotrypsin

TABLE XI

VALUES OF MOLECULAR WEIGHTS AND STOKES RADII OF MARKER PROTEINS

Marker proteins	Molecular weight (kDa)	Stokes radius (nm)
BSA dimer[a]	135	4.3
BSA monomer[b]	69	3.5
Ovalbumin[b]	43	3.0
Pepsin[a]	35.5	2.29
Chymotrypsinogen - A[b]	25.7	2.2
Cytochrome c[b]	12.4	1.7

References:- [a] Andrews (1970)

[b] Khan (1982)

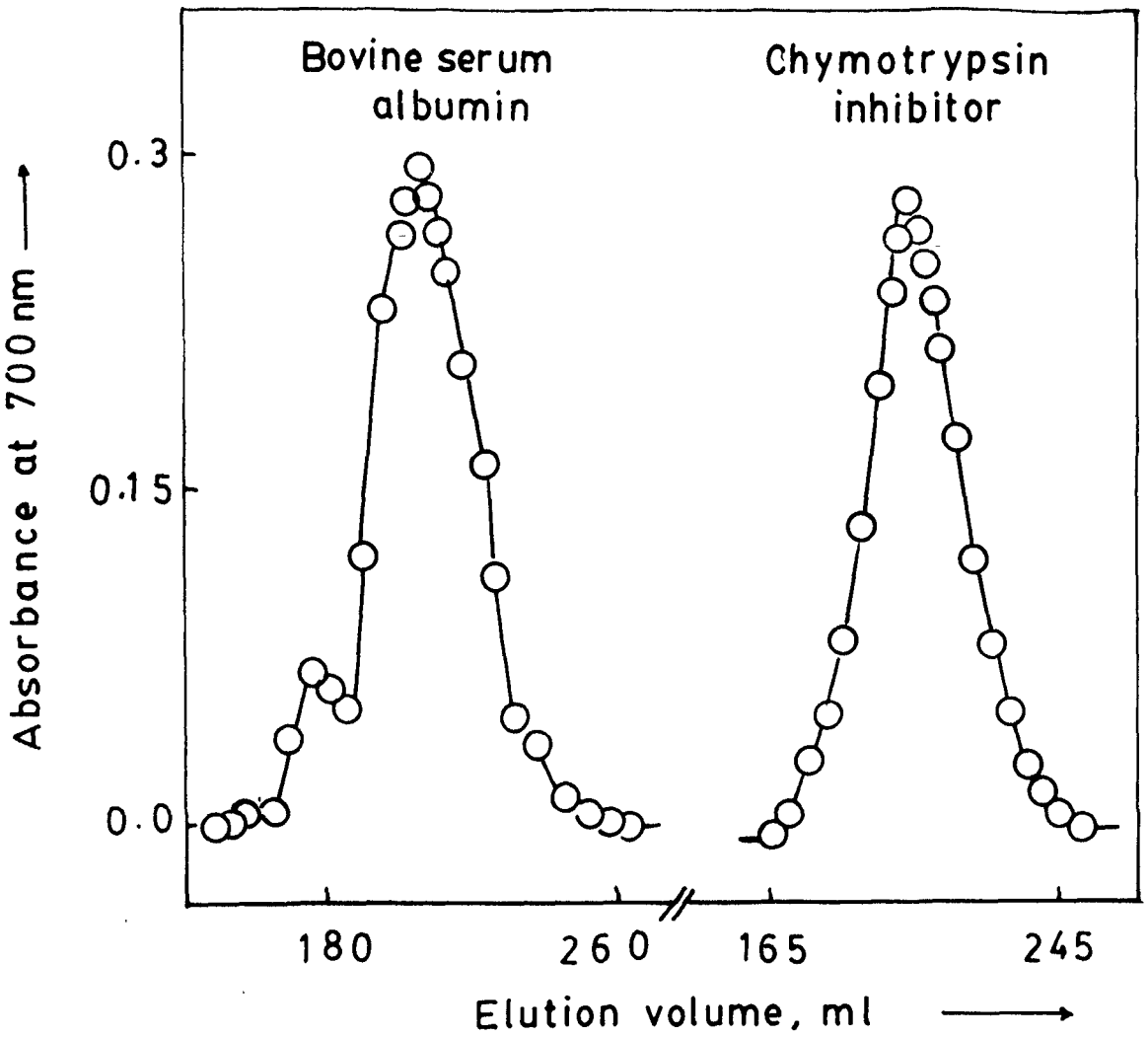


Fig.22. Elution profiles of bovine serum albumin and chymotrypsin inhibitor on Sephadex G - 200 column.

About 10 mg of protein was applied on Sephadex G - 200 column(2.4 × 78 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02 % sodium azide. The protein was eluted in 2 - 5 ml fractions at a flow rate of 20 ml / hr. The column was monitored by the method of Lowry et al., (1951).

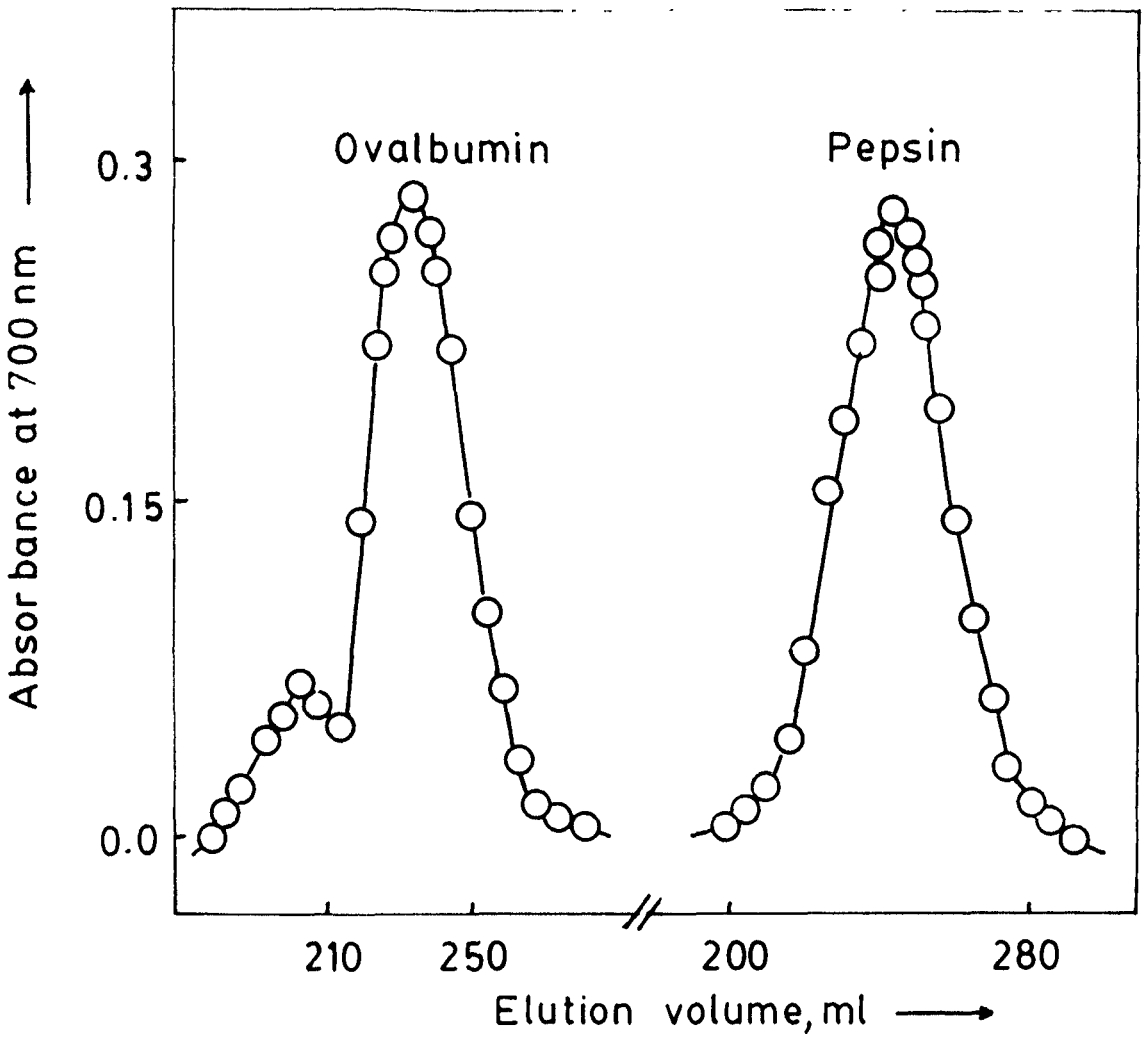


Fig. 23. Elution profiles of ovalbumin and pepsin on Sephadex G-200 column.

The experimental conditions are same as given in legend of Fig. 22.

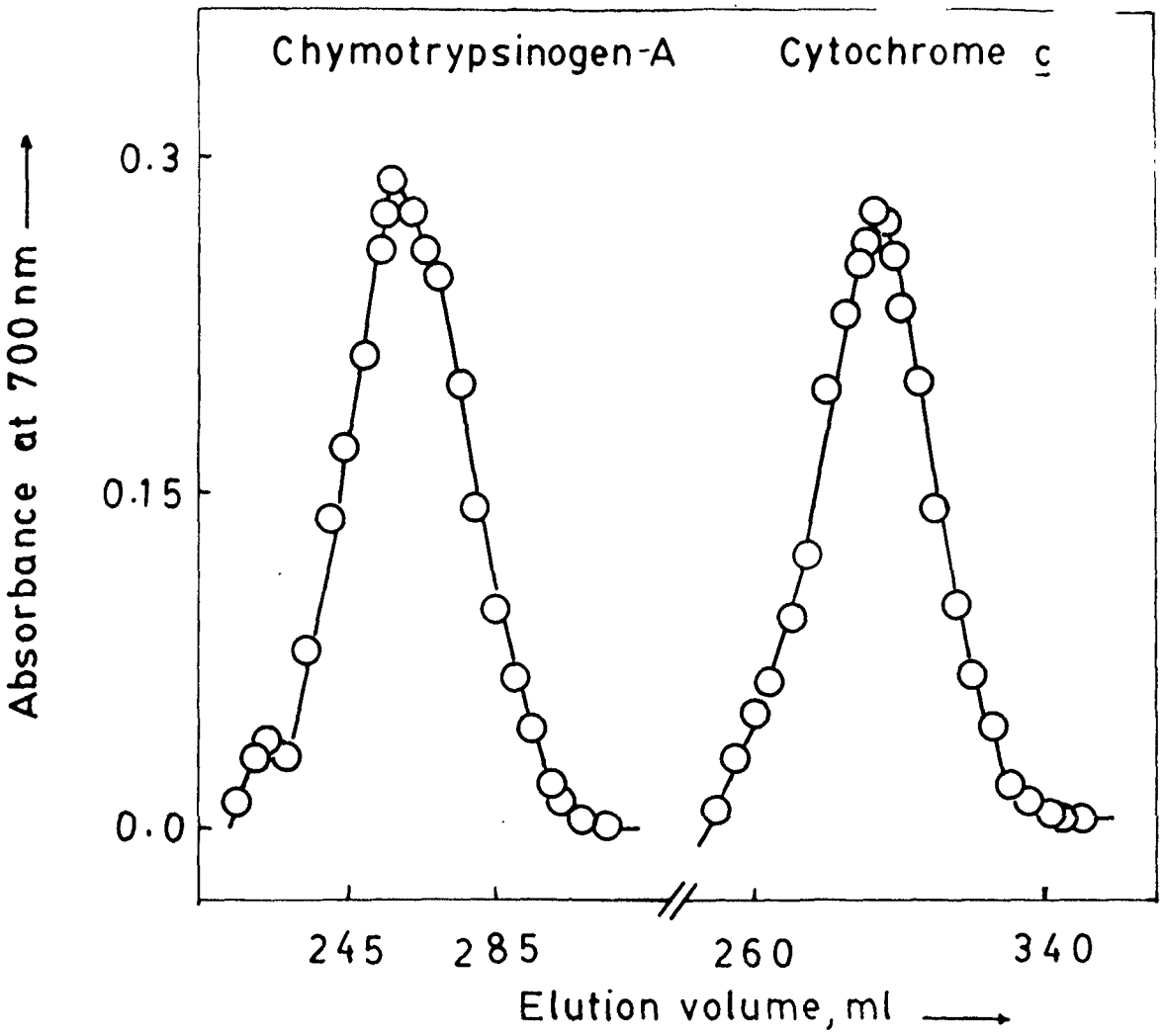


Fig. 24. Chromatographic profiles of chymotrypsinogen A and cytochrome c on Sephadex G – 200 column.

The experimental conditions are same as given in legend of Fig. 22.

TABLE XII

LOG M, V_e/V_o, $M^{1/3}$ AND $K_d^{1/3}$ VALUES OF THE MARKER PROTEINS AND CHYMOTRYPSIN INHIBITOR USED IN GEL FILTRATION EXPERIMENTS

Proteins	V_e/V_o	Log M	$K_d^{1/3}$	$M^{1/3}$
BSA dimer	1.25	5.1303	0.56	51.2993
BSA monomer	1.46	4.8389	0.69	41.0157
Ovalbumin	1.67	4.6335	0.78	35.0334
Pepsin	1.74	4.5502	0.8	32.8657
Chymotrypsinogen A	1.85	4.4099	0.84	29.5106
Cytochrome c	2.1	4.0934	0.92	23.1459
Chymotrypsin Inhibitor	1.44	-	0.68	-

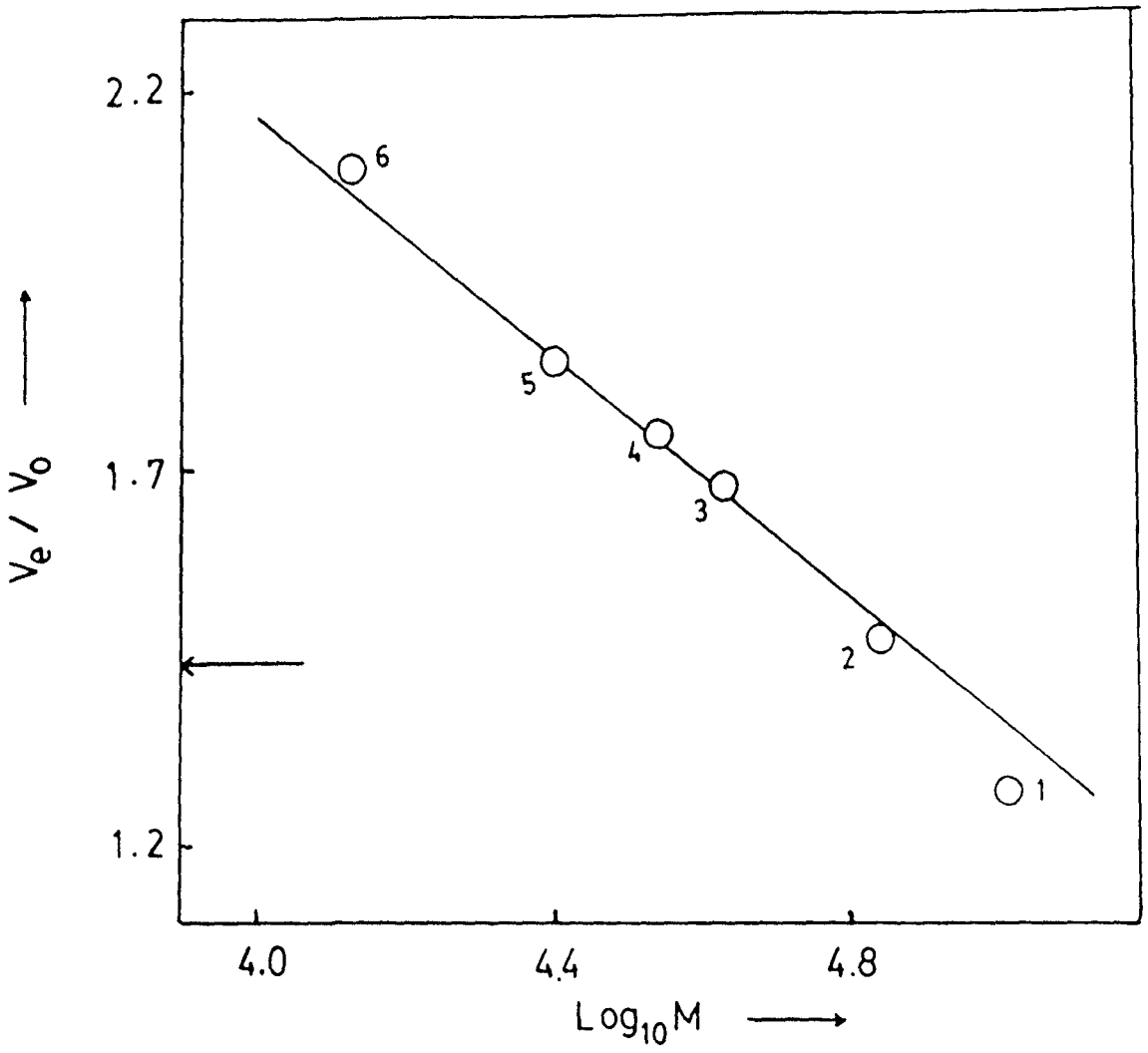


Fig. 25. Plot of gel filtration data according to Andrews (1970).

The marker proteins used were: 1. BSA dimer, 2. BSA monomer, 3. ovalbumin, 4. pepsin, 5. chymotrypsinogen A, 6. cytochrome c. The value of V_e / V_o for chymotrypsin inhibitor is shown by an arrow. The linear plot obtained by the method of least square fits the equation :-

$$V_e / V_o = (0.82) \log_{10} M + 5.45$$

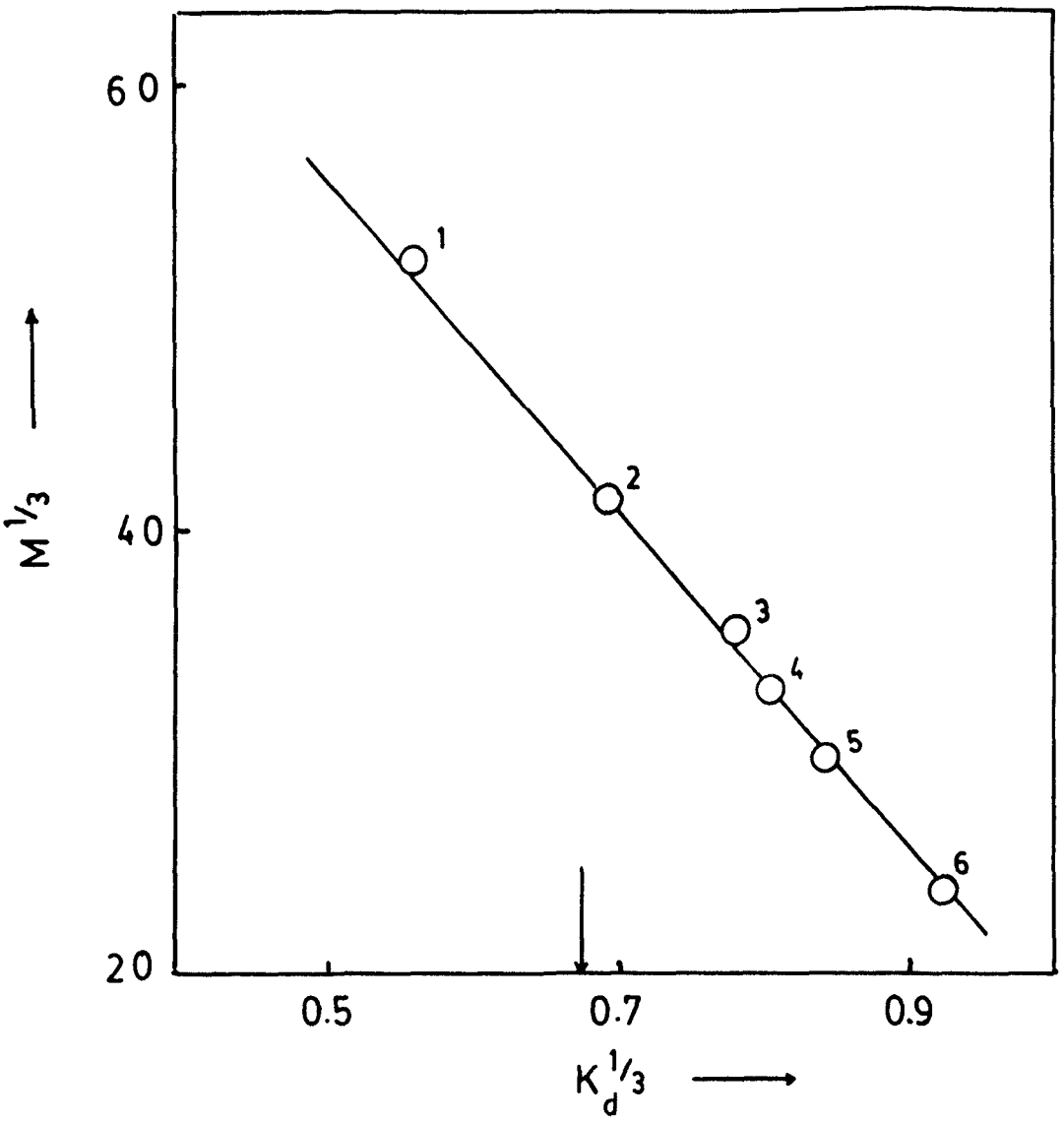


Fig.26. Analysis of gel filtration data according to Porath (1963).

The marker proteins used were: 1 BSA dimer, 2. BSA monomer, 3 ovalbumin, 4. pepsin, 5. chymotrypsinogen A, 6. cytochrome c. The value of $K_d^{1/3}$ for chymotrypsin inhibitor is shown by an arrow. The straight line computed by the help of least square analysis, fits the equation :-

$$M^{1/3} = (-78.01)\ K_d^{1/3} + 95.03$$

inhibitor from these two values was calculated as 76 kDa which was 12% higher than that obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The values of K_d, K_{av} and other related parameters were calculated for marker proteins and as well as for chymotrypsin inhibitor and the results are summarized in Table XIII. To determine Stokes radius of inhibitor, the gel filtration data were analyzed according to Ackers (1967) using the following expression:-

$$r = 3.64 \ (\ erfc^{-1}K_d) + 0.93 \qquad (14)$$

where $erfc^{-1}K_d$ is the error function complement of distributioin factor of protein with Stokes radius of r. A least square analysis of a plot of $erfc^{-1}K_d$ versus r yielded a straight line as shown in Fig. 27. The value of $erfc^{-1}K_d$ for chymotrypsin inhibitor was computed to be 0.72 which according to equation (14) would correspond to a Stokes radius of 3.55 nm.

Another plot was drawn according to Laurent and Killander (1964) between Stokes radius and $(-\log K_{av})^{1/2}$ and is shown in Fig. 28. The straight line obtained by least square analysis fits the following equation:-

TABLE XIII

GEL FILTRATION DATA FOR THE MARKER PROTEINS AND THE CHYMOTRYPSIN INHIBITOR ON SEPHADEX G - 200 COLUMN

Proteins	K_d	$erfc^{-1}K_d$	K_{av}	$(- \log K_{av})^{1/2}$
BSA dimer	0.175	0.95	0.164	0.89
BSA monomer	0.32	0.7	0.301	0.72
Ovalbumin	0.47	0.51	0.441	0.6
Pepsin	0.52	0.45	0.488	0.56
Chymotrypsinogen A	0.595	0.38	0.559	0.5
Cytochrome c	0.77	0.21	0.72	0.38
Chymotrypsin inhibitor	0.31	0.72	0.291	0.73

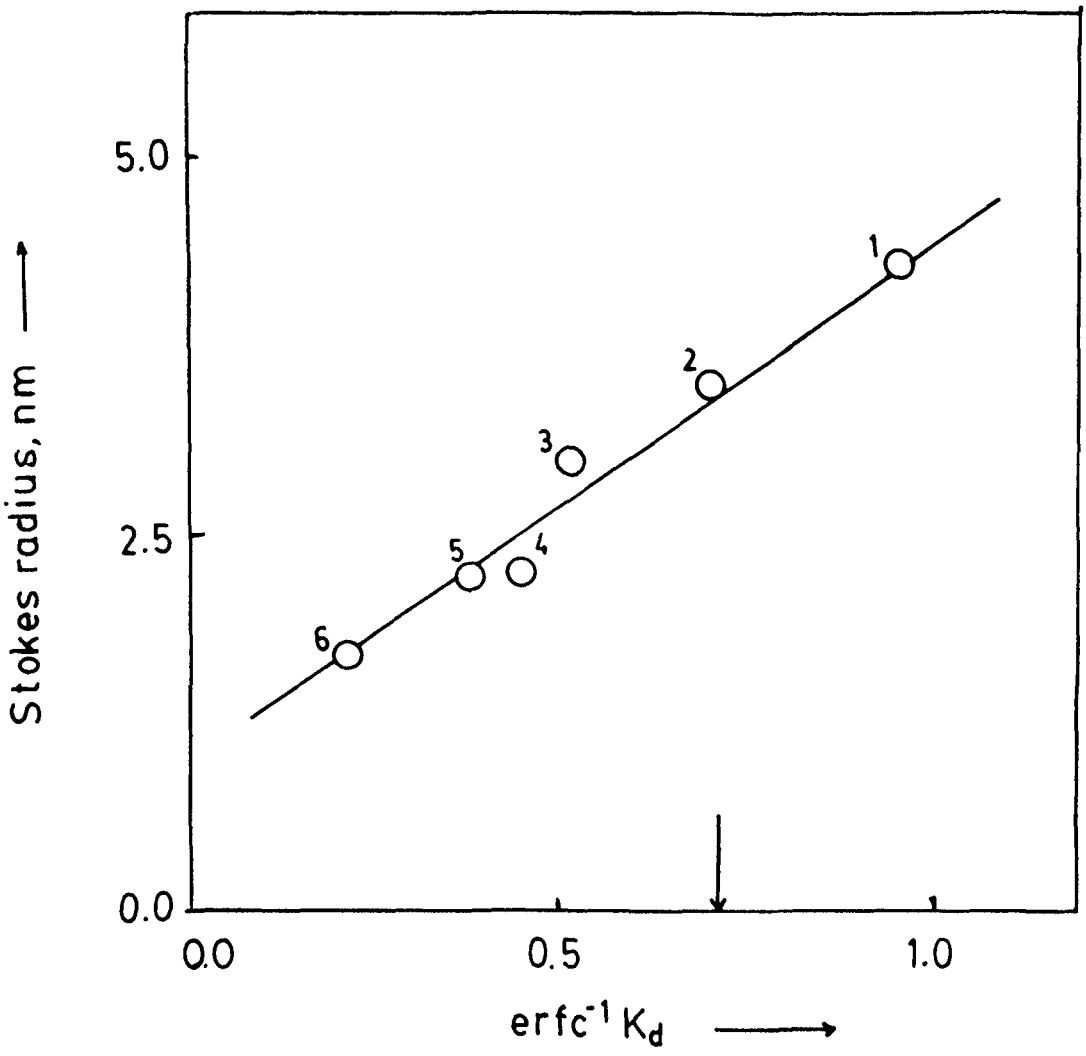


Fig.27. Plot of Stokes radius versus $erfc^{-1} K_d$ obtained on a Sephadex G-200 column.

The marker proteins used were: 1. BSA dimer, 2. BSA monomer, 3. ovalbumin, 4. pepsin, 5. chymotrypsinogen A, 6. cytochrome c. The value of $erfc^{-1}K_d$ for chymotrypsin inhibitor is indicated by an arrow.

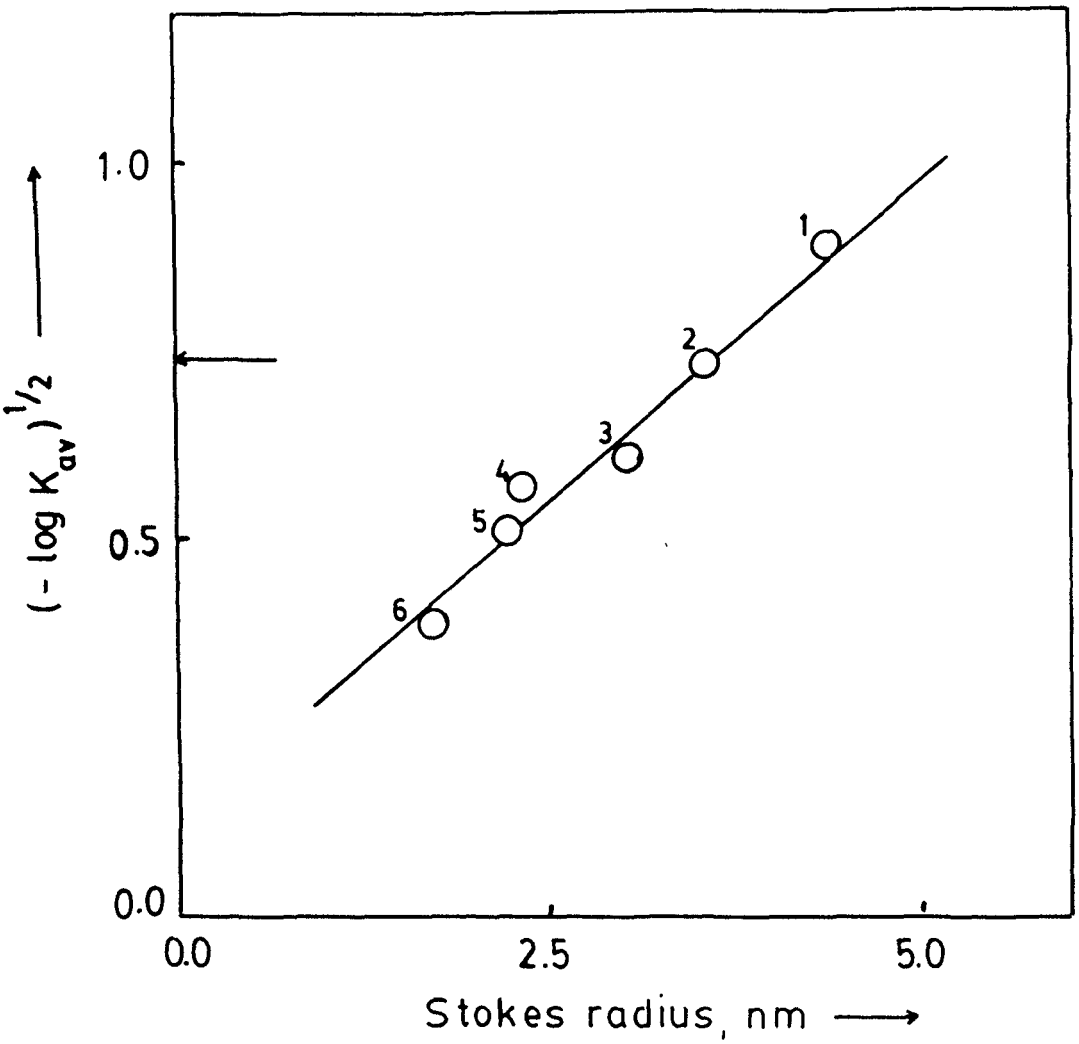


Fig. 28 Plot of $(-\log K_{av})^{1/2}$ values of marker proteins versus Stokes radius.

The marker proteins used were: 1. BSA dimer, 2 BSA monomer, 3 ovalbumin, 4 pepsin 5 chymotrypsinogen A, 6 cytochrome c. The value of $(-\log K_{av})^{1/2}$ for chymotrypsin inhibitor is shown by an arrow.

$$(- \log K_{av})^{\frac{1}{2}} = 0.182\ r + 0.092 \qquad (15)$$

The value of $(- \log K_{av})^{\frac{1}{2}}$ of the inhibitor was determined as 0.73 which according to equation (15) would give the value of 3.51 nm for the Stokes radius of the inhibitor.

From the knowledge of Stokes radius diffusion coefficient, D, of the inhibitor was calculated with the help offollowing expression (Andrews, 1970):--

$$D = k\ T\ /\ 6\ \pi \eta\ r \qquad (16)$$

Where k is the Boltzmann constant, η is the coefficient of viscosity of the medium (i.e. 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride) and T is the absolute temperature. With k = 1.386×10^{-16} ergs/degree; η = 0.01 poise; T = 302 ^{o} K and the determined value of Stokes radius, the value of diffusion coefficient for the inhibitor was calculated as 6.29×10^{-7} cm^2/sec.

The frictional ratio, f/f_o, of the inhibitor was calculated using the relation (Siegel and Monty, 1966):--

$$f/f_o = r/(3\ \bar{v}_2\ M/4\ \pi\ N\ )^{1/3} \qquad (17)$$

where N is Avogadro's number (6.023×10^{23} per mole) and $\bar{v}_2$ is the partial specific volume of protein in ml/g . The partial specific volume of goat chymotrypsin inhibitor was taken to be 0.715 ml/g , same as that of human $alpha_1$-antichymotrypsin calculated from amino acid and carbohydrate composition (Travis et al., 1978b). The molecular weight of inhibitor was taken as 68 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Using these values, the frictional ratio for chymotrypsin inhibitor was calculated as 1.32 with the help of equation (17).

6. **Functional properties:**

The activity of chymotrypsin inhibitor was measured against both chymotrypsin and trypsin in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. It was found to inhibit both the enzymes, however, inhibition was found to be more pronounced for chymotrypsin than trypsin.

Chymotrypsin was first passed on a Sephadex G - 200 column to enrich the preparation and then it was treated with TLCK. On treatment with TLCK 10% of the total enzyme activity was reduced which may be due to the

contamination of trypsin in our chymotrypsin preparation. The TLCK treated chymotrypsin was then taken for further studies.

The stoichiometry of the reaction between chymotrysin/trypsin and inhibitor was determined by treating a constant amount of enzyme with varying concentrations of inhibitor and the results are summarized in Figs. 29 and 30. Evidently complete inhibition of chymotrypsin was achieved at an inhibitor to enzyme molar ratio of 1.1 (see Fig. 29), however, this ratio increased to 4.54 for trypsin.

For studies on the time course of inactivation of chymotrypsin by the goat chymotrypsin inhibitor, an aliquot of 1 ml sample was drawn at different time intervals from an enzyme and inhibitor mixture and its residual caseinolytic activity measured. The results on the inactivation of chymotrypsin and trypsin are respectively shown in Figs. 31 and 32, where it can be seen that the inhibition of chymotrypsin was completed within 5 minutes. In contrast, the inhibition of trypsin by goat chymotrypsin inhibitor was a slow process and required about 50 minutes to achieve complete inhibition. It is, thus clear that the inhibitor abolished the chymotryptic

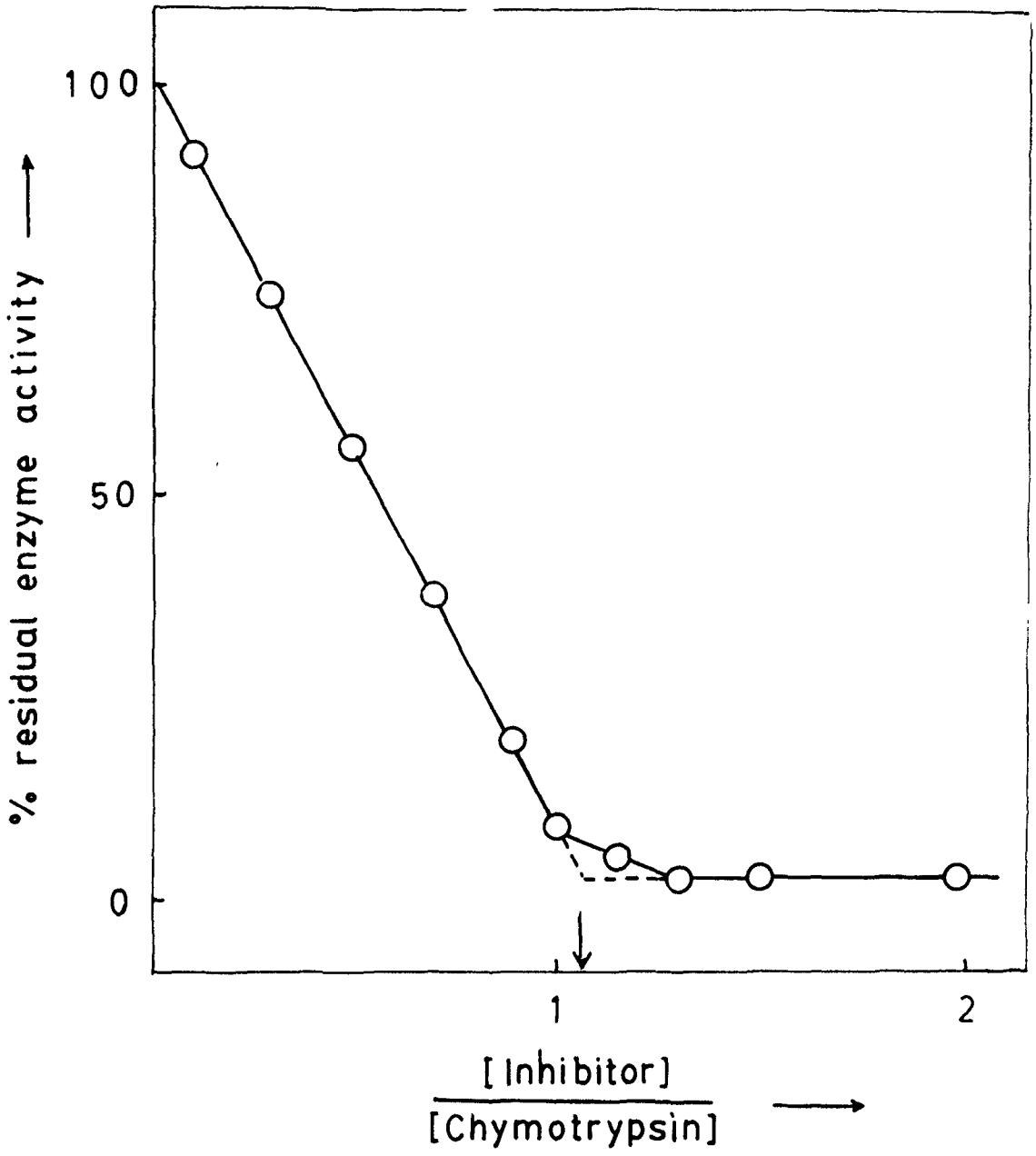


Fig. 29. Effect of inhibitor concentration on its activity.

The enzyme, chymotrypsin (4.35×10^{-6} mM) was incubated with differerent concentration of chymotrypsin inhibitor (4.35×10^{-7} mM to 8.7×10^{-6} mM) for 20 minutes at 37 °C. The residual enzyme activity was measured according to the method of Anson (1938) using casein as substrate.

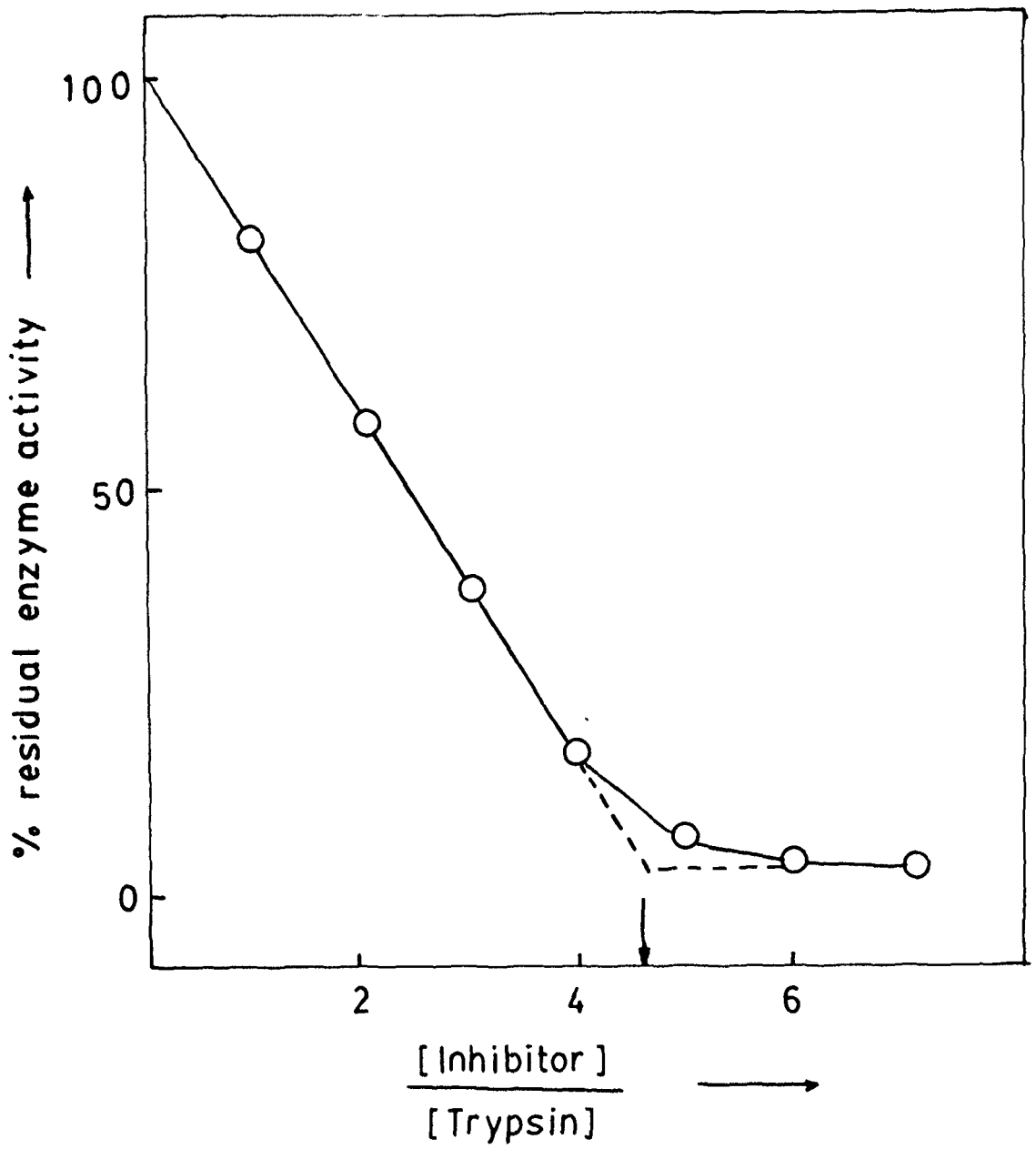


Fig.30. Inhibition of trypsin by chymotrypsin inhibitor.

Increasing amount of chymotrypsin inhibitor (4.29×10^{-6} mM to 3×10^{-5} mM) were added to constant amounts of trypsin (4.29×10^{-6} mM) and incubated for 50 minutes at 37°C. The residual enzyme activity was measured using casein as substrate.

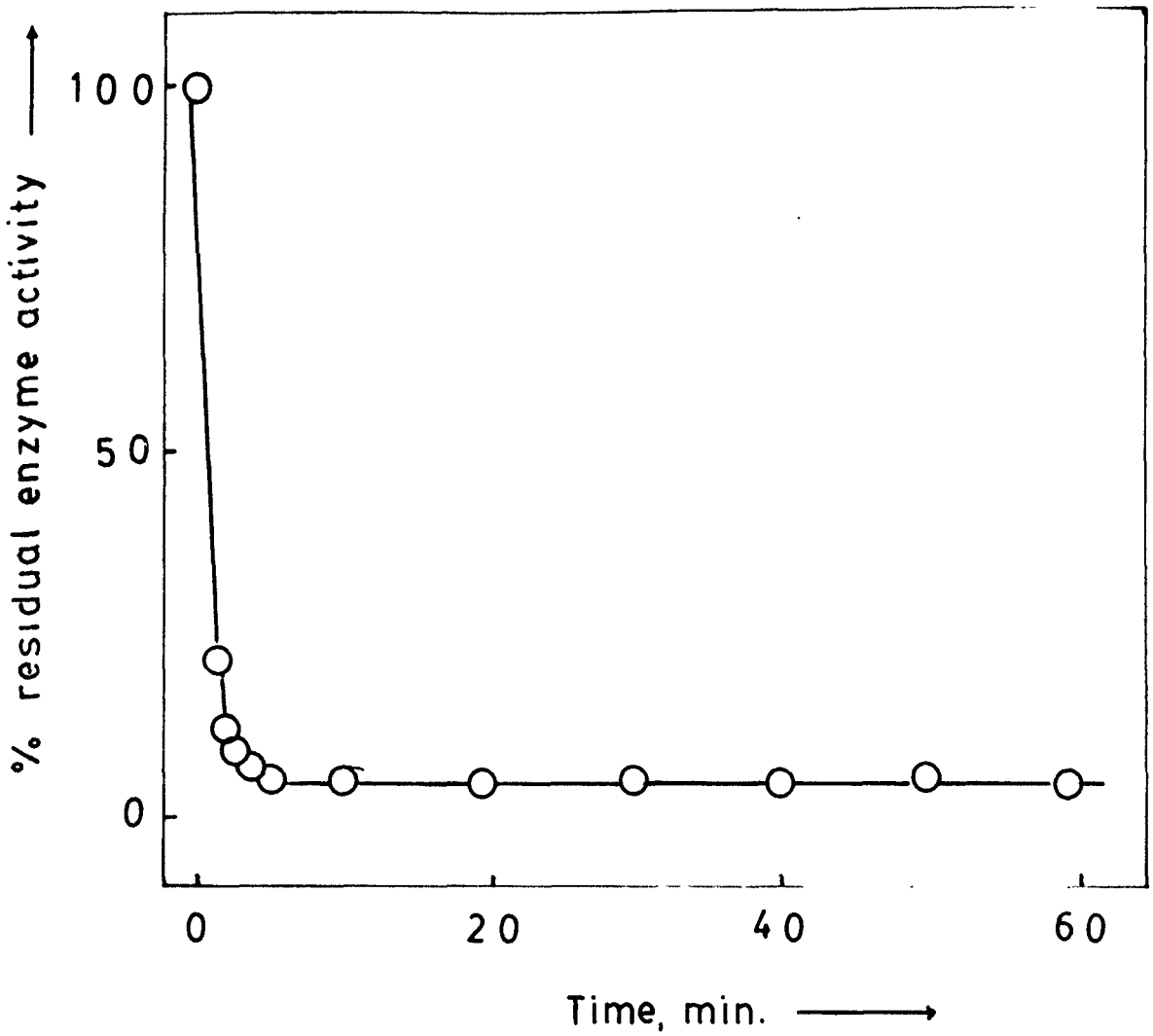


Fig. 31. A plot of residual enzyme activity as a function of incubation time of chymotrypsin / chymotrypsin inhibitor mixture.

Residual enzyme activity was measured on aliquots withdrawn at different time intervals from a mixture of inhibitor/chymotrypsin (molar ratio 1:1) kept at 37°C. The residual enzyme activity was measured using casein as substrate.

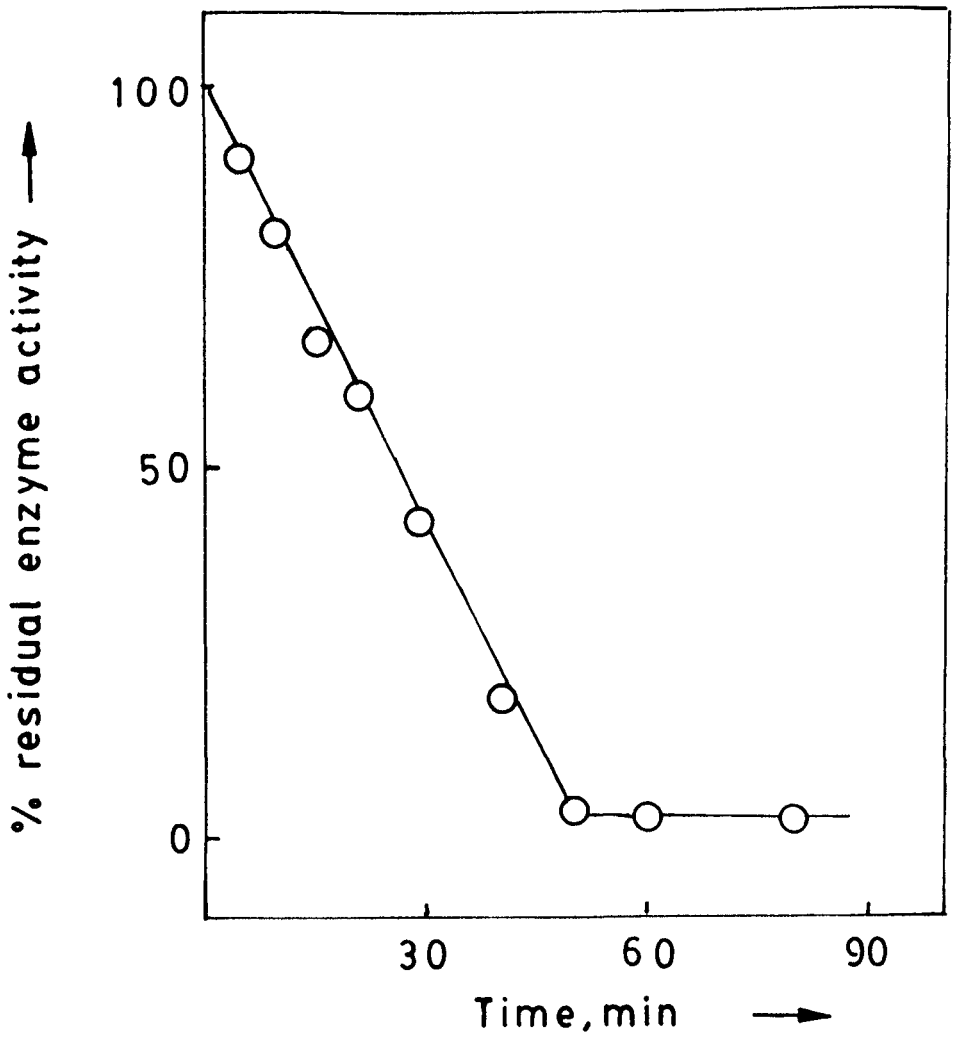


Fig. 32. **Residual enzyme activity versus incubation time of chymotrypsin inhibitor with trypsin.**

Residual enzyme activity was measured on aliquots withdrawn at different time intervals from a mixture of inhibitor and enzyme (molar ratio 5:1) kept at 37°C for 50 minutes. The residual enzyme activity was measured using casein as substrate.

activity in a biologically feasible time. It is, therefore, possible to analyse results of Fig. 31 with the help of the following expression (Beith, 1980):--

$$1/[E] = k_{ass}\ t + 1/[E]_o \qquad (18)$$

where $[E]_o$ is the enzyme concentration at time zero, [E] is the enzyme concentration at any time, t. The basic assumption involving the above expression is that the inhibition should be irreversible one for which the only relevant rate constant will be k_{ass}. This is likely to be true because most of the plasma proteinase inhibitors are proteolytically modified upon reaction with proteinases (Laine et al., 1982a) and the resultant inhibitor enzyme complexes are usually highly stable (Travis and Salvesen,1983). Secondly, the enzyme and the inhibitor should be in equal concentrations.

A plot of 1/[E] versus t should be linear according to equation (18). The value of [E] was determined from the dependence of caseinolytic activity on total enzyme concentration. Such a plot was obtained from the data of Fig. 31 by the method of least square and is shown in Fig. 33. From the slope of the linear plot,the

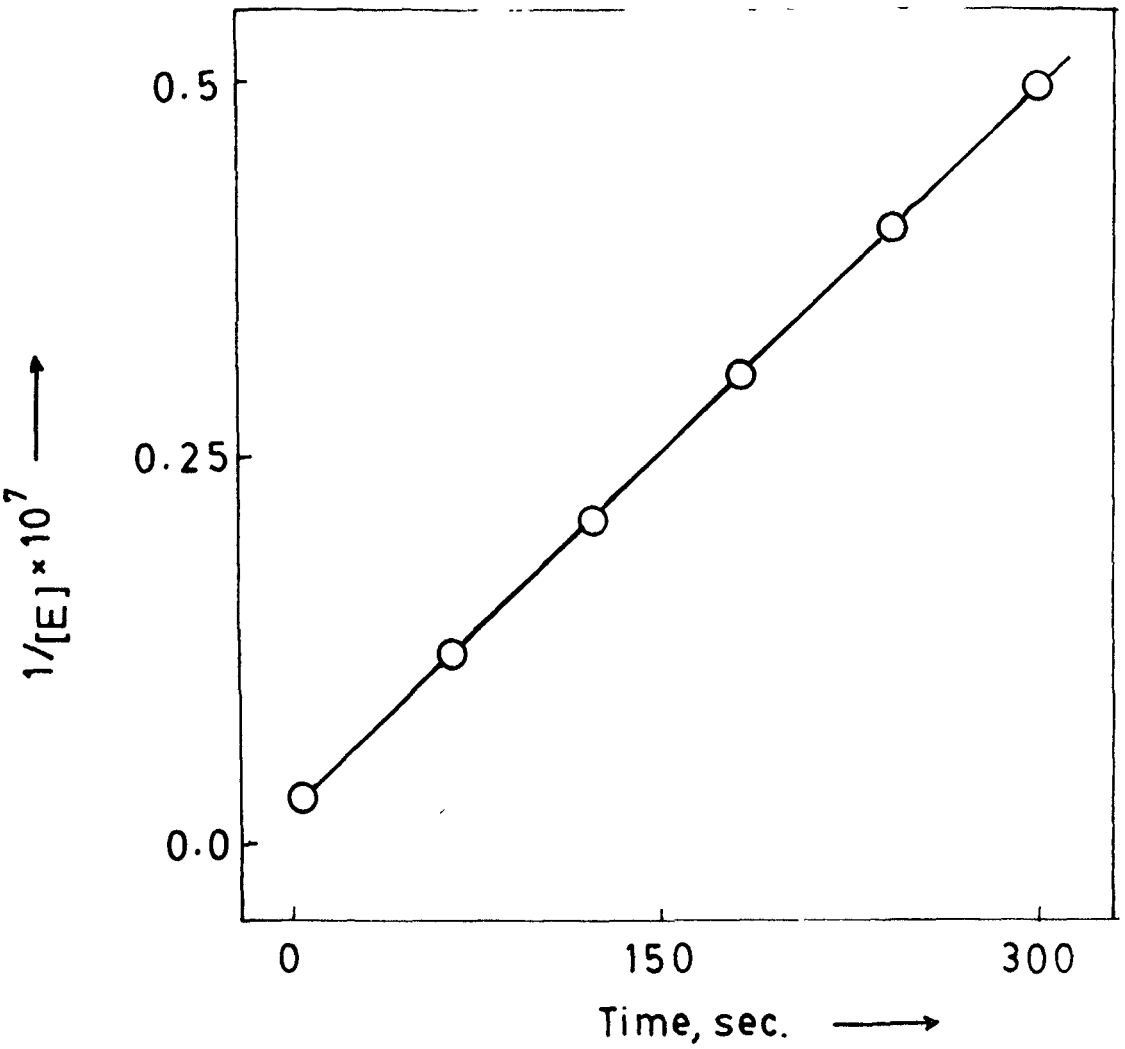


Fig.33. The second order plot of time dependency of the inhibition of chymotrypsin by goat chymotrypsin inhibitor in accordance with equation (18).

rate constant of association of the inhibitor with the enzyme i.e. k_{ass} was calculated to be 1.6×10^4 M^{-1} s^{-1}. The interaction of human $alpha_1$-antichymotrypsin with human and bovine chymotrypsin has been studied (Beatty et al., 1980) and the reported value ($1 - 6 \times 10^4$ M^{-1} s^{-1}) was found to be comparable to the one obtained in this study.

7. **Effect of heat treatment on antichymotryptic activity of chymotrypsin inhibitor:**

Goat chymotrypsin inhibitor was heated at 45°C, 50°C , 55°C and 60°C for 15 minutes in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. No insoluble protein was visible in the solution. The decrease in the inhibitory activity of the goat chymotrypsin inhibitor at different temperatures is depicted in Fig. 34. The chymotrypsin inhibitor treated at 45°C retained the same inhibitory activity as that of native inhibitor. When the inhibitor was heated at 50°C and 55°C, the losses in inhibitory activity at these temperatures were found to be 35% and 65% respectively. The inhibitor completely lost its activity when heated at 60°C . It is noteworthy that the human $alpha_1$-antichymotrypsin was reported to be stable upto 50°C

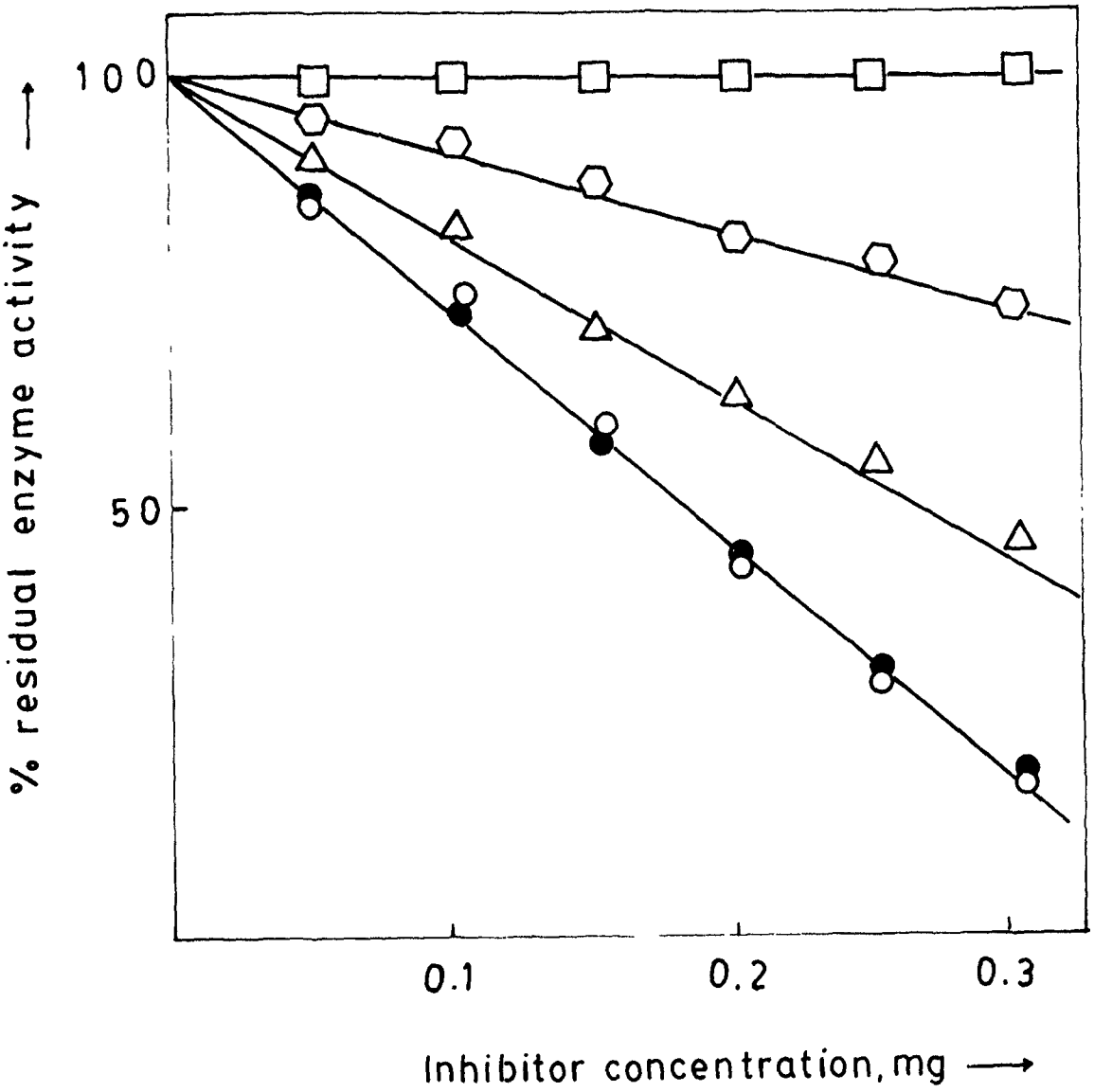


Fig. 34. Effect of heat treatment on the activity of chymotrypsin inhibitor.

A fixed concentration of chymotrypsin (4.29×10^{-6} mM) and an indicated concentration of native inhibitor (—○—○—) or inhibitor which was heated at 45°C (—●—●—), 50°C (—△—△—), 55°C (—⬡—⬡—) and 60°C (—□—□—) was incubated at 37°C for 30 minutes. The residual enzyme activity was measured using casein as substrate as described in the text.

while the activity was reduced to half when it was heated at 55°C for 15 minutes and 60°C treated inhibitor completely lost its activity (Matsumoto et al., 1982b; Tsuda et al., 1986).

DISCUSSION

Chymotrypsin inhibitor was isolated both from goat plasma and serum by ammonium sulphate fractionation at pH 4.5 followed by purification by ion exchange chromatography. Interestingly, the inhibitor thus isolated was found to be free from extrenous proteins as judged by single coomassie stainable protein band in sodium dodecyl sulphate polyacrylamide gel electrophoresis in Tris glycine buffer (25 mM Tris and 194 mM glycine), pH 8.3. Salt fractionation at pH 4.5 resulted in the removal of major plasma protein viz, albumin which could not be removed to the desired extent when ammonium sulphate fractionation was carried out at pH 7.0. In this respect, the present procedure differs from other methods used earlier for the isolation of $alpha_1$-antichymotrypsin from human plasma/serum (Travis et al., 1978b; Siddiqui et al., 1980; Abdullah et al., 1983; Laine et al., 1984b) In previous methods after salt fractionation usually more than one step of purification was required for isolating human plasma $alpha_1$-antichymotrypsin of comparable purity (Travis et al., 1978b; Abdullah et al., 1983). Further, unlike the procedure used by Laine et al., (1984b) in which human serum $alpha_1$-antichymotrypsin was exposed to

pH 2.8 during isolation, the present method does not involve the exposure of goat chymotrypsin inhibitor to pH less than 4.5. This is important in view of the notable observation made by Travis et al., (1978a) who found that the human $alpha_1$-antichymotrypsin was 80% inactivated when exposed to pH 3.0 for 5 minutes.

Goat chymotrypsin inhibitor in this study was found to be a sialoglycoprotein containing 12% neutral hexose and 4.5% sialic acid residues. This would correspond to 47 moles of neutral hexose and 10 moles of sialic acid residues per mole of inhibitor; the molecular weight used in the calculation was 68 kDa. For human $alpha_1$-antichymotrypsin Heimburger et al., (1971) and later Travis et al., (1978b) found 38-41 neutral hexose and 11-14 sialic acid residues per mole of protein. However, Laine and Hayem (1981) reported that both human serum and pleural fluid inhibitors have same amount of neutral hexoses and sialic acid residues; the values being 35 and 10 residues respectively per mole of inhibitor. In contrast to these inhibitors, chymotrypsin inhibitor isolated from other sources such as marine turtle egg white (Guha and Sinha, 1984) and silkworm larval hemolymph (Sasaki, 1985) do not contain any

carbohydrate. This observation is noteworthy since survival of the inhibitor in the serum depends on the carbohydrate content as it was found that half life of rat $alpha_1$-proteinase inhibitor without carbohydrate is six times less than that for the inhibitor with carbohydrate (Weber et al., 1985).

The inhibitor under native state contains one free sulfhydryl group which is available for the reaction with p-hydroxymercuribenzoate. However, the fact that the treatment with p-hydroxymercuribenzoate did not abolish the activity of goat chymotrypsin inhibitor, excludes any functional role for the lone sulfhydryl group of the inhibitor. Such studies have not been carried out for other inhibitors. It will be, therefore, interesting to determine free sulfhydryl group of chymotrypsin inhibitor present in other mammalian serum including human serum.

The molecular weight of goat chymotrypsin inhibitor was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis to be 68 kDa both under reducing and non-reducing conditions suggesting that inhibitor is a single chain protein molecule.

Similar values (64-68 kDa) were reported for human alpha$_1$-antichymotrypsin both by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Travis et al., 1978b; Siddiqui et al., 1980) and sedimentation equilibrium (Travis et al., 1978b; Katsunuma et al., 1980). Since the goat chymotrypsin inhibitor has been found to be a sialoglycoprotein containing 17% carbohydrate, the molecular weight estimated by gel filtration is expectedly higher i.e. 76 kDa. This observation is in agreement with the results obtained in this laboratory by Waheed and Salahuddin (1975). After exclusion of carbohydrate moieties, the molecular weight of human alpha$_1$-antichymotrypsin was calculated to be 44 kDa (Matsumoto et al., 1982b; Chandra et al., 1983) which is identical to that (43 kDa) determined for chymotrypsin inhibitor isolated from silkworm larval hemolymph (Sasaki, 1985). It will be, therefore, interesting to find out structural differences between silkworm larval hemolymph chymotrypsin inhibitor and mammalian inhibitor. However chymotrypsin inhibitor ·olated from other sources such as ascaris (Babin et

al., 1984), snake venom (Ritonja, 1983b) and marine turtle egg white (Guha and Sinha, 1984) are usually low molecular weight proteins ranging between 7-13 kDa. Here it should be pointed out that by sodium dodecyl sulphate polyacrylamide gel electrophoresis Laine and Hayem (1981) obtained significantly lower value (58 kDa) for molecular weight of human $alpha_1$-antichymotrypsin isolated from pleural fluid. It seemed that inhibitor in pleural fluid might be destroyed by some proteases but they have obtained the same results also for serum $alpha_1$-antichymotrypsin (Laine and Hayem, 1981; Laine et al., 1984b). Thus this may be either due to different calibrations and conditions used in sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laine and Hayem, 1981) or due to exposure of inhibitor to acidic pH (Berninger, 1985).

The isoionic pH of the goat inhibitor has been found in this study to be 5.5 at a protein concentration of 1.6%. The corresponding value for human $alpha_1$-antichymotrypsin is not available. However, thin-layer polyacrylamide gel isoelectric focussing of human $alpha_1$-antichymotrypsin showed seven bands with isoelectric points at pH values of 4.1. 4.15, 4.2, 4.26, 4.32,

4.38 and 4.45. The microheterogeneity of $alpha_1$-antichymortypsin persisted even after removal of sialic acid by neuraminidase treatment. The different isotypes of human $alpha_1$-antichymortypsin persisted even after removal of sialic acid by neuraminidase treatment. The different isotypes of human $alpha_1$-antichymotrypsin interacted normally with chymotrypsin (Gianazza and Arnaud, 1981). The ultra-violet light absorption spectra of chymotrypsin inhibitor studied in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride, showed maximum absorption near 278 nm where the specific extinction coefficient was determined to be 6.23 $cm^2 g^{-1}$; the value of specific extinction coefficient at 280 nm was 5.92 $cm^2 \ g^{-1}$. The value of specific extinction coefficient at 280 nm is similar to that (6.2 $cm^2 \ g^{-1}$) measured for human $alpha_1$-antichymotrypsin (Travis et al., 1978b). Available data on human $alpha_1$-antichymotrypsin showed the presence of 3-4 tryptophan residues, 9-10 tyrosine residues and 24-26 phenylalanine residues per mole of inhibitor (Heimburger et al., 1971; Travis et al., 1978b; Chandra et al., 1983) In view of these considerations it seems that goat

chymotrypsin inhibitor is also a tryptophan containing protein. This contention finds further support from the study of excitation and emission spectra of inhbitor under native conditions (Fig.18), where it can be seen that fluorescence emissioin for the inhibitor was maximum at 338 nm; the wavelength of excitation was 278 nm.

The gel filtration behaviour of goat chymotrypsin inhibitor was studied on Sephadex G-200 column and its Stokes radius was determined as 3.53 nm. The diffusion coefficient (6.29×10^{-7} cm^2/sec.) and frictional ratio, f/f_o (1.37) determined for the inhibitor showed that goat chymotrypsin inhibitor possesses a nonglobular conformation. Alternatively the hydrodynamic data particularly the deviation of f/f_o from unity can be interperated in terms of unusual high hydration of the inhibitor. It appears to be a real possibility in view of the fact that the inhibitor conotains 17% carbohydrate which would extensively interact with water molecules.

Results presented on the functional properties of goat chymotrypsin inhibitor in this thesis showed that goat inhibitor is an effective inhibitor of chymotrypsin

and abolishes all chymotryptic activity at a molar ratio of enzyme to inhibitor of 1.1. The inactivation at this molar ratio was rather rapid. However, it showed weak inhibitory activity against trypsin. The inhibition of tryptic activity was slow and required substantially higher inhibitor concentration ([I]/[E] = 4.5) for achieving complete inhibition. Our results also suggest that association constant, k_{ass}, for inhibitor-chymotrypsin system is about 1.57×10^4 M^{-1} s^{-1}. Further goat chymotrypsin inhibitor shows significant thermal stability. It retains almost all inhibitory activity upon heating at 45°C. However, heat treatment above 45°C resulted in the loss of activity. It is interesting to note that the values of k_{ass} for the interaction of human alpha$_1$-antichymotrypsin with human chymotrypsin (1.0×10^4) and bovine chymotrypsin (6.0×10^4) were similar within experimental error with that (1.5×10^4) determined in this study (Beatty et al., 1980). The thermal stability of human alpha$_1$-antichymotrypsin was also found to be similar (Matsumoto et al., 1982b; Tsuda et al., 1986). Strikingly, human alpha$_1$-antichymotrypsin did not show any detectable activity against trypsin (Travis et al., 1983a). In contrast goat chymotrypsin

inhibitor showed measurable although weak antitryptic activity. In conclusion our results showed that the goat chymotrypsin inhibitor is not identical to human plasma/serum alpha$_1$-antichymotrypsin.

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LIST OF PRESENTATIONS

1. "Isolation of $alpha_1$-antichymotrypsin from human plasma"
   Renu Tyagi

   Proceedings of the 53rd Annual Meeting of the Society of Biological Chemists (India) held at Delhi from October 12-14, 1984.

2. "Studies on $alpha_1$-antichymotrypsin"
   Renu Tyagi

   Proceedings of the 54th Annual Meeting of the Society of Biological Chemists (India) held at Pantnagar from November 7-9, 1985.

3. "Isolation and purification of $alpha_1$-antichymotrypsin from goat plasma"
   Renu Tyagi

   Proceedings of the 56th Annual Meeting of the Society of Biological Chemists (India) held at Tirupathi from December 28-30, 1987.

4. "Studies on goat chymotrypsin inhibitor"

   Renu Tyagi

   Proceedings of the 57th Annual meeting of the Society of Biological Chemists (India) held at Delhi from October 9-12, 1988.